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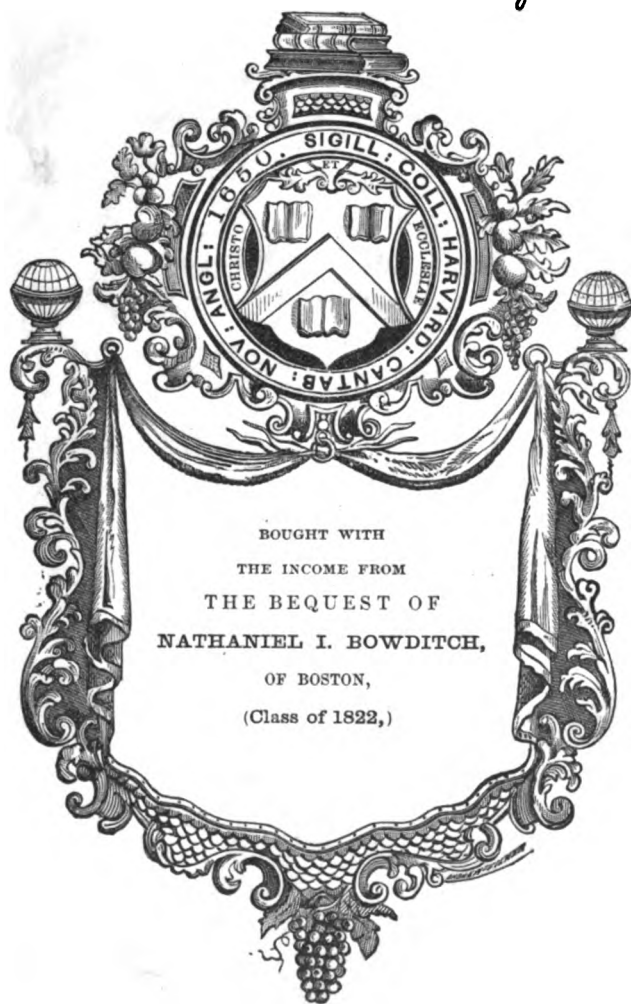
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The Journal of Infectious Diseases

FOUNDED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

Supplement No. 1, May, 1905

EDITED BY

LUDVIG HEKTOEN AND EDWIN O. JORDAN

IN CONJUNCTION WITH

FRANK BILLINGS

F. G. NOVY

W. T. SEDGWICK

*Some of the Papers presented to the Laboratory Section
of the American Public Health Association
at the Havana Meeting,
January 9, 1905*



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The Journal of Infectious Diseases

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Supplement No. 1, May, 1905

REPORT OF COMMITTEE ON STANDARD METHODS OF WATER ANALYSIS TO THE LABORATORY SECTION OF THE AMERICAN PUBLIC HEALTH ASSOCIATION.

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LETTER OF TRANSMITTAL

NEW YORK, December 19, 1904.

To the Chairman and Members of the Laboratory Section of the American Public Health Association.

GENTLEMEN: The final report of your Committee on Standard Methods of Water Analysis is submitted herewith.

It is now more than ten years since there arose in North America a movement for securing the adoption of more uniform and efficient methods for water analysis, particularly of bacteriological methods. Earlier accomplishments¹ related largely to uniform methods of recording chemical results. The late Dr. Wyatt Johnston of Montreal was the first one to call the attention of the Association to this line of work, and at the Montreal meeting in 1894 the first step² in this direction was taken. By invitation of a subcommittee of the Committee on Pollution of Water Supplies, a convention³ of American bacteriologists assembled in New York in June, 1895, and appointed a committee to draw up procedures for the study of bacteria in a uniform manner and with special reference to the differentiation of species. This committee submitted a report⁴ at the Philadelphia meeting of the Association in 1897. It was published early in 1898 and has been widely used in various laboratories in

this country. The demand for copies of it still continues, although for some time it has been out of print.

At the Minneapolis meeting in 1899 the present committee was appointed with a view to extending the standard procedures to include not only the determinations of species of bacteria, but all the other lines of investigation involved in the analysis of water.

The committee undertook first to ascertain the views of the analysts of America regarding not only the bacteriological, but also the chemical, physical and microscopical examinations of water. Circular letters were sent to all the principal laboratories, and much coöperative work was done in connection with the differentiation of species of bacteria.

Progress reports were made at meetings held at Indianapolis, Buffalo and New Orleans in 1900, 1901 and 1902, the two latter being published in the *Proceedings of the Association* for those years, and the former in *Science*.⁵

In 1901 this committee was instructed to revise the 1897 report of the Bacteriological Committee in order to exclude from it those features not found to have been of general service and to include such new matter as later developments had justified.

In 1902 the committee suffered a severe loss through the death of one of its most valued members, the late Dr. Wyatt Johnston of Montreal, who is credited by all with the initiation of this movement, Dr. Adolph Gehrmann of Chicago resigned from the committee in that year. At the New Orleans meeting the full membership of the committee was restored by the appointment of Mr. R. S. Weston of Boston and Mr. J. W. Ellms of Cincinnati.

The past decade has been a transitional period for water analysis. As this field of sanitary investigation has been extended to cover in a more thorough manner the conditions existing throughout this country, it has been found that the methods applicable to the fairly clear waters of the Atlantic seaboard and to many European waters have not been adequate for the analysis of the muddy waters of the South and West. Conversely, new methods used in certain sections of the country are for similar reasons not always applicable to the conditions existing where the earlier studies were made.

The purification of water supplies has received a great impetus during the past decade, and the water analyst is more and more being placed in charge of the operation of large public filter plants. In work of this character, especially when muddy waters are being treated, he thus uses some methods of analysis which are not generally required for sanitary work; while, on the other hand, he omits with propriety various tests which form an essential part of the customary sanitary analysis.

The presence of objectionable amounts of iron in various ground water supplies and the introduction of special processes for its removal have in some instances severely taxed the resources of the water analyst, and necessitated modifications of old methods.

Methods for determining lead and copper are being used with more frequency than was the case in earlier years, when less attention was given to all the aspects of public health.

Treatment of sewage, to prevent gross nuisances and to prevent the pollution and infection of streams, has recently received a marked stimulus through the development of the so-called rapid biological filters, the septic

tank treatment, etc. Special tests and analyses have been required in connection with these lines of work—notably those for the determination of the putrescibility of the effluents of sewage works. The study of the longevity of disease germs in connection with the question of infection of water supplies drawn from streams at various distances below the discharge of outfall sewers has brought into prominence new lines of bacteriological investigation.

Enough has been said in outline of the new and varied requirements made of the water analyst to show the need of a broad and substantial basis for his methods and for his work under present conditions. Some of the older methods, used for the study of the general sanitary quality of unfiltered water supplies, are becoming less and less important, while the newer ones, used in the operation of purification plants, are becoming of more value. This is in keeping with the modern tendency of the analyst to become more and more an important factor in connection with the operation of plants for the purification of water and sewage. Indeed, unpurified sources of water supply are becoming fewer and fewer, as hygienic demands are being met by the rapid introduction of purification works, as evidenced by the best practice both in Europe and in America.

The methods of analysis presented in this report as “standard methods” are believed to represent the best current practice of American water analysts, and to be generally applicable in connection with the ordinary problems of water purification, sewage disposal and sanitary investigations. Analysts working on widely different problems manifestly cannot use methods which are identical, and special problems obviously require the methods best adapted to them; but, while recognizing these facts, it yet remains true that sound progress in analytical work will advance in proportion to the general adoption of methods which are reliable, uniform and adequate.

It is said by some that standard methods within the field of applied science tend to stifle investigation, and that they retard true progress. If such standards are used in the proper spirit this ought not to be so. The committee strongly desires that every effort shall be continued to improve the technique of water analysis, and especially to compare current methods with those herein recommended, where different, so that the results obtained may become still more accurate and reliable than they are at present.

In Table No. 1 are given the more essential determinations which, in the opinion of the committee, should be applied to each of the principal lines of analytical work in connection with the ordinary problems of water supply and sewage disposal. It is realized that some of the older laboratories are hardly in a position at the present time to follow out these suggestions in a literal manner, although on new work it is believed that they could follow them to advantage.

Some of these suggestions may seem radical, and in special instances they may be indeed inexpedient, as already mentioned, but on the whole it is believed that they indicate the lines along which the water analyst may direct his efforts to best advantage and with the feeling that he is obtaining all the data necessary, while doing little or nothing that is needless.

It will be noted that the bacteriological determinations, including the tests for *Bacillus coli*, are given much more prominence than was for-

merly the case, and that less attention is given to the organic matter as determined by chemical analysis. This is because of the inability of the chemical methods to separate that portion of the organic matter which is of no sanitary significance from that which is associated with pollution or infection.

The most substantial steps in advance relate to improvements in the physical and chemical methods required in connection with the operation of plants for the improvement and purification of water supplies.

Detailed descriptions of the various methods recommended are given in concise form, covering the essential features of each determination. It is assumed that those using these directions are thoroughly grounded in the fundamental principles of chemistry and biology, and that they are also familiar with the leading literature upon the subject. So many satisfactory textbooks upon chemical analysis in general and on water analysis in particular are in existence that it is unnecessary to give a complete detailed description of all procedures; but it is fully recognized that in many cases the adherence to certain details is an essential matter, and hence for the newer methods they are incorporated in this report.

Some of the methods described are known in different parts of America by different proper names, hence it has been the endeavor of the committee to describe them with sufficient clearness to make plain what procedures are meant without reference to the name of the author, but to give due credit for the method by referring to his published work in the bibliography at the end of the report.

The bibliography is by no means a full list of important works on water analysis. It is simply a list of references to the works most consulted in America, arranged for the purpose of assisting the reader in getting in touch with the general aspects of a method, including its history and application, together with full technical details of the procedure as now practiced.

No attempt is made to report upon the interpretation of the results of water analyses, or upon the classification of bacteria, as these subjects are receiving the attention of other committees of the Association,

This report does not deal with any of the numerous phases of applied bacteriology in the domains of medicine or industrial science. It is hoped, however, that workers in these fields may find useful portions dealing with the preparation of media, and that published descriptions of bacteria associated with disease or with various industrial processes will be made to conform with the procedures herein recommended.

Very respectfully,

(Signed) GEORGE W. FULLER, *Chairman.*
GEORGE C. WHIPPLE, *Secretary.*
H. W. CLARK.
EDWIN O. JORDAN.
H. L. RUSSELL.
J. W. ELLMS.
ROBERT SPURR WESTON.

[illegible]

* For observations and comments see next page.

OBSERVATIONS ON TABLE 1.

Plus (+) sign indicates that the test is advised for each type of water under which it appears; while a zero (0) indicates that the test is not advised.

This table indicates the general views of the committee, and is not intended to limit the use of other tests, either specially or regularly, where the local conditions show such supplementary tests to be desirable.

In some instances the tests need not be made on all samples, such, for example, as in the case of dissolved oxygen, and putrescibility of sewage before treatment. The plus signs are here used to show that the information conveyed by these tests should be known to the analyst; and that it is unsafe to assume that all sewages are putrescible or lacking in dissolved oxygen during all hours of the day. Turbidity, free carbonic acid, alkalinity and sulphate contents at intervals are also valuable in sewage work.

Free mineral acids and sulphates are important data for surface waters in the coal regions.

Tests for lead, copper, tin, and zinc are frequently called for in studying the effect of waters upon service pipes.

Whenever chemicals are applied to water for any purpose, the results of analyses should record the disposition of the applied chemicals. Water treated by mechanical filters should be tested frequently for acidity, that is, undecomposed coagulant. Softened waters should be tested for caustic alkalinity in a thorough manner as detailed under hardness.

INTRODUCTION TO THE 1897 REPORT OF THE BACTERIOLOGICAL COMMITTEE.

As explained by Dr. Smart in the preface to this report, a convention of bacteriologists from the United States and Canada assembled in the city of New York, on June 21 and 22, 1895, in response to the invitation of a sub-committee of the Committee on the Pollution of Water Supplies of the American Public Health Association. The proceedings of this convention, including the papers read and their discussion, were published in the *Journal of the American Public Health Association*, October, 1895. These papers and discussions related mainly to technical procedures to be followed in the systematic study of bacteria, with especial reference to their description and identification. There was general agreement of opinion as to the importance of securing greater precision and uniformity in the methods of studying and describing bacterial species. A committee of members of the convention was therefore appointed to prepare a report, to be presented to the Water Committee of the American Public Health Association, this report to contain recommendations concerning bacteriological methods based partly upon the deliberations of the convention and partly upon a wider study of the subject. The members selected for this committee were Drs. J. George Adami, William T. Sedgwick, George W. Fuller, Charles Smart, Alexander C. Abbott, T. M. Cheesman, Theobald Smith, and William H. Welch.

A first draft of a report was drawn up by Dr. Adami and submitted to the members of the committee, who made various suggestions. The final preparation of the report was undertaken by Dr. T. M. Cheesman, Instructor in Bacteriology in the College of Physicians and Surgeons, Columbia University, New York. The following statement by Dr. Adami well expresses the aims and manner of preparation of the report:

Naturally, with a committee, the members of which are so widely scattered, it has been found impossible to hold frequent meetings, but at these meetings the members have found themselves singularly in accord upon everything

relating to the main points at issue. Naturally, also, correspondence and the circulation of the report in its various stages have not been found entirely satisfactory in eliciting the opinions of every member upon matters of detail. But all these means accomplished much, and it was eventually found possible to place the final drafting of the recommendations in the hands of one member. We cannot sufficiently express our indebtedness to Dr. Cheesman for the amount of time, and indeed of independent work which he has devoted to this task.

The recommendations thus do not indicate the previous procedure in all details of any single member of the committee, but are a concord of what has appeared to be the best in the methods and technique of all the members and of bacteriologists generally. To have indicated in the following pages wherein any single member found himself unable to accept in its entirety any one of the many recommendations would have counteracted our main object, that, mainly, of inducing uniformity and precision in procedure in the study and descriptions of species. Each member, therefore, to attain this object has voluntarily refrained from demanding that one or other method, to which from long employment he has become firmly attached, should be inserted in these pages. The committee freely admits that there may be other and better methods than those here detailed. It has, on the other hand, striven to recommend what in the present state of our knowledge would seem to be the best and most likely to gain acceptance. It does not demand of bacteriologists in general — it does not promise for its own members in particular — that these and only these methods shall be employed. It does but ask that *where new species are being studied for publication* the procedure here recommended be given a trial, and that, for the direction of other workers, where it has been employed a note be given to that effect, e. g., "cultures in broth (Method B. C.) presented the following characters:" — or, "save where otherwise indicated, the B. C. methods have been used."

In short, the committee recognizes fully that these recommendations must of necessity be provisional. It publishes them in the hope that by this act it will direct attention to the urgent need now existing for full and accurate descriptions of species of bacteria in which the items have been determined by methods common to the main body of workers, and as a consequence are capable of verification and control.

The report is not intended to be a complete treatise upon bacteriological technique. Its purpose is to make certain recommendations concerning methods to be pursued in the study of bacteria, with the view of securing greater uniformity and exactness in the determination and description of the characters of bacterial species. When one considers the difficulty, often the impossibility, of the identification of many bacterial species or varieties described in literature, in consequence of imperfections and carelessness in the determination and description of their characters, it is evident that the attainment of the purpose aimed at in this report is greatly to be desired.

The report deals especially with certain ordinary and fundamental procedures in bacteriological technique, and it does not attempt to cover fully the entire field. In a science so rapidly developing as bacteriology, it need scarcely be said that any attempt to present the best technical procedures can apply only to the existing state of the science, and that much will be added and much corrected in the near future. It is hoped that the recommendations in this report may prove useful to workers in bacteriology, and especially may lead to greater accuracy and fullness and uniformity in the determination and description of the characters of bacteria.

(Signed) WILLIAM H. WELCH.

PREFACE TO THE 1897 REPORT OF THE BACTERIOLOGICAL COMMITTEE.

At the meeting of the American Public Health Association in Montreal, Canada, in 1894, the committee on the Pollution of Water Supplies closed its report with the suggestion of a coöperative investigation into the bacteriology of water supplies as a means of bringing order out of the chaotic state of the literature of water bacteria, and of throwing light from the bacteriological side on questions of practical sanitation. This suggestion was approved by the Association and the Chairman of the Committee was authorized to build up a committee for collective bacteriological investigation. The bacteriologists promptly acceded to the proposition. They recognized that such an investigation would give an immense impetus to bacteriological work; that it would do much to clear away the confusion surrounding species, and to increase and systematize our knowledge; and that practical results might also be expected, particularly as regards the typhoid and colon bacilli, the unwholesomeness of water supplies and the means of lessening the prevalence of typhoid fever and diarrheal diseases. A sub-committee consisting of Professor J. George Adami, Dr. Wyatt Johnston, Mr. George W. Fuller and myself, appointed to determine the methods of laboratory procedure to be adopted by the committee in the practical work of the investigation, found it impossible to formulate a satisfactory scheme of work until certain questions, mostly relating to technique, had been discussed fully and settled in accordance with the most advanced knowledge of the subjects concerned. An effort to effect this by correspondence developed so much variation in the practice of the different laboratories that it became needful to call a convention for a thorough discussion of the points at issue. The convention was held in the Academy of Medicine, New York City, June 21 and 22, 1895. Most of the prominent bacteriologists of the United States and Canada were present, but although the members were informed beforehand of the subjects that were to be brought up for settlement, and although full discussion was given to each under the chairmanship of Professor Welch of Johns Hopkins University, many of the points presented so much difficulty that the whole series was referred to a committee, with the understanding that the convention would accept its decision.

This committee consisted of

J. George Adami, McGill University, Chairman.
A. C. Abbott, University of Pennsylvania.
T. M. Cheesman, College Physicians and Surgeons, New York.
George W. Fuller, Louisville Water Company.
W. T. Sedgwick, State Board of Health, Massachusetts.
Charles Smart, U. S. Army.
Theobald Smith, Harvard University.
W. H. Welch, Johns Hopkins University.

The committee met in New York City in February 1896 to digest its material and outline its report which was presented to the American Public Health Association at its meeting in Buffalo, New York, in September of that

year. The report was subsequently withdrawn for further criticism and amendment, and was finally submitted for publication at the meeting of the Association in Philadelphia, Pennsylvania, September, 1897.

(Signed) CHARLES SMART.

ACKNOWLEDGMENTS.

During the five years that the Committee on Standard Methods of Water Analysis has been in session, it has been in correspondence with most of the leading bacteriologists and sanitary chemists of America, and many of them have engaged in the practical coöperative work of comparing methods. To all of those who have thus aided in this work the Committee wishes to express its appreciation and thanks. The following is a list of those who deserve special recognition:

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Jordan, E. O.	- - -	Chicago, Ill.
McFarland, J. W.	- - -	Philadelphia, Pa.
Park, W. H.	- - -	New York, N. Y.
Randolph, R. B. F.	- - -	Trenton, N. J.
Russell, H. L.	- - -	Madison, Wis.
Walters, E. P.	- - -	Boston, Mass.
Wesbrook, F. F.	- - -	Minneapolis, Minn.
Whipple, George C.	- - -	New York, N. Y.

COLLECTION OF SAMPLES.

QUANTITY OF WATER REQUIRED FOR ANALYSIS.

The minimum quantity necessary for making the ordinary physical, chemical, and microscopical analyses of water or sewage is one gallon; for the bacteriological examination, two ounces. In special cases larger quantities may be required.

BOTTLES.

The bottles for the collection of samples shall be made of hard, clear, white glass, and shall have glass stoppers. Cork stoppers shall not be permitted except when physical or microscopical examinations only are to be made. Earthen jugs or metal containers shall not be used.

Sample bottles shall be carefully cleansed each time before using. This may be done by treating with sulphuric acid and potassium bichromate, or with alkaline permanganate and afterwards with a mixture of oxalic and sulphuric acids, and by thoroughly rinsing with water and draining.

When clean, the stoppers and necks of the bottles shall be protected from dirt by tying cloth or thick paper over them.

For shipment they shall be packed in cases with a separate compartment for each bottle. Wooden boxes may be lined with indented fibre paper, felt, or some similar substance, or provided with spring corner strips, to prevent breakage. Lined wicker baskets also may be used.

Bottles for bacterial samples, besides being washed, shall be sterilized with dry heat for one hour at $160^{\circ}\text{C}.$, or in an autoclave at $115^{\circ}\text{C}.$ for fifteen minutes. For transportation they may be wrapped in sterilized cloth or paper, or the necks may be covered with tin-foil and the bottles put in tin boxes. When bacterial samples must of necessity stand for twelve hours before plating, bottles holding more than four ounces shall be used.

The bottles used for chemical samples may be sterilized and the samples so collected used for the bacteriological analysis. When bacterial samples are not plated at the time of collection they shall be kept on ice at a temperature of not more than $15^{\circ}\text{C}.$, and preferably as low as $10^{\circ}\text{C}.$

TIME INTERVAL BETWEEN COLLECTION AND ANALYSIS.

Generally speaking, the shorter the time elapsing between the collection and the analysis of a sample, the more reliable will be the analytical results. Under many conditions, analyses made in the field are to be commended, as data so obtained are frequently preferable to those made in a distant laboratory after the composition of the water has changed en route.

The allowable time that may elapse between the collection of a sample and the beginning of its analysis cannot be stated definitely, as it depends upon the character of the sample and upon other conditions, but the following may be considered as fairly reasonable maximum limits under ordinary conditions:

Physical and Chemical Analysis.

Ground waters	-	-	-	-	-	-	-	72 hours
Fairly pure surface waters	-	-	-	-	-	-	-	48 "
Polluted surface waters	-	-	-	-	-	-	-	12 "
Sewage effluents	-	-	-	-	-	-	-	6 "
Raw sewages	-	-	-	-	-	-	-	6 "

Microscopical Examination.

Ground waters	- - - - -	72 hours
Fairly pure surface waters	- - - - -	24 "
Waters containing fragile organisms	-	Immediate examination

Bacteriological Examination.

Ground waters	- - - - -	6 hours
Fairly pure surface waters	- - - - -	6 "
Polluted surface waters	- - - - -	6 "
Sewage effluents	- - - - -	Immediate plating
Raw sewages	- - - - -	" "

If sterilized by the addition of chloroform, formaldehyde, mercuric chloride, or some other disinfectant, samples for chemical and microscopical examination may be allowed to stand for longer periods than those indicated, but as this is a matter which must vary according to local circumstances, no definite procedure is recommended.

If unsterilized samples of sewage, sewage effluents, and highly polluted surface waters are not analyzed on the day of their collection, caution must be used in regard to the organic contents, which frequently change materially upon standing.

The gaseous contents of samples, especially dissolved oxygen, and carbonic acid should be obtained immediately, in accordance with the directions given beyond in connection with each determination.

REPRESENTATIVE SAMPLES.

Care shall be taken to secure a sample which is truly representative of the liquid to be analyzed. In the case of sewages this is especially important, in view of the marked variations in composition which occur from hour to hour. Frequently satisfactory samples can be obtained only by mixing together several portions collected at different times or at different places—the details as to collection and mixing depending upon local conditions.

PHYSICAL EXAMINATION.**TEMPERATURE.**

The temperature of the sample shall be taken at the time of collection, and shall be preferably expressed in Centigrade degrees, to the nearest 0.5 degree or closer if for any reason more

exact data are required. For obtaining the temperature of water at various depths below the surface the thermophone⁶ is recommended.

TURBIDITY.

The turbidity of water is due to suspended matter, such as clay, silt, finely divided organic matter, microscopic organisms, etc. The increasing use of filters for the purification of water and sewage has made this determination one of great importance.

TURBIDITY STANDARD.

The standard of turbidity shall be that adopted by the United States Geological Survey, namely, a water which contains 100 parts of silica per million in such a state of fineness that a bright platinum wire one millimeter in diameter can just be seen when the center of the wire is 100 millimeters below the surface of the water and the eye of the observer is 1.2 meters above the wire, the observation being made in the middle of the day, in the open air, but not in sunlight, and in a vessel so large that the sides do not shut out the light so as to influence the results. The turbidity of such water shall be 100.

COEFFICIENT OF FINENESS.⁷

The number obtained by dividing the weight of suspended matter in the sample (in parts per million) by the turbidity shall be called the coefficient of fineness. If greater than unity it indicates that the matter in suspension in the water is coarser than the standard; if less than unity, that it is finer than the standard.

PREPARATION OF SILICA STANDARD.⁸

Use diatomaceous earth as free as possible from sponge spicules and amorphous silica. Wash with water to remove soluble salts; dry, and ignite to remove organic matter; treat and warm with dilute hydrochloric acid; wash with distilled water until free of acid, and dry thoroughly.

Grind in an agate mortar, sifting through a No. 200 mesh sieve in order to separate mats obtained by grinding; and dry in a desiccator.

One gram of this preparation in one liter of distilled water makes a stock suspension which contains 1000 parts per million of

silica, and which should have a turbidity of 1000. Test this suspension, after diluting a portion of it with nine times its volume of distilled water, with a wire to ascertain if the silica has the necessary degree of fineness, and if the suspension has the necessary degree of turbidity. If not, correct by adding more silica or more water as the case demands.*

Standards for comparison shall be prepared from this stock suspension by dilution with distilled water. For turbidity readings below 20, standards of 0, 5, 10, 15 and 20 shall be kept in gallon bottles made of clear white glass; for readings above 20, standards of 20, 30, 40, 50, 60, 70, 80, 90 and 100 shall be kept in 100 c.c. nessler tubes, approximately 20 millimeters in diameter.

Comparison of the water under examination with the standards shall be made by viewing them sidewise toward the light, looking at some object and noting the distinctness with which the margins of the object can be seen.

The standards shall be kept stoppered, and both sample and standards shall be thoroughly shaken before making the comparison.

In order to prevent any bacterial or algal growths from appearing in the standards, a small amount of bichloride of mercury may be added to them.

PLATINUM WIRE METHOD.⁹

This method requires a rod with a platinum wire of a diameter of one mm. or 0.04 inch inserted in it about one inch from the end of the rod, and projecting from it at least one inch at a right angle. Near the end of the rod, at a distance of 1.2 meters (about four feet) from the platinum wire, a wire ring shall be placed directly above the wire through which, with his eye directly above the ring, the observer shall look when making the examination. The rod shall be graduated as follows:

The graduation mark of 100 shall be placed on the rod at a distance of 100 mm. from the center of the wire. Other graduations shall be made according to Table 2, which is based on the best obtainable data and in which the distances are intended to be such that when the water is diluted the turbidity readings will

*This method of correction very slightly alters the coefficient of fineness of the standard, but does not noticeably affect its use.

decrease in the same proportion as the percentage of the original water in the mixture. These graduations are those used to construct what is known as the U. S. Geological Survey Turbidity Rod of 1902.¹⁰

TABLE 2.

Turbidity. Parts per Million	Vanishing Depth of Wire mm.	Turbidity. Parts per Million	Vanishing Depth of Wire mm.
7	1095	70	138
8	971	75	130
9	873	80	122
10	794	85	116
11	729	90	110
12	674	95	105
13	627	100	100
14	587	110	93
15	551	120	86
16	520	130	81
17	493	140	76
18	468	150	72
19	446	160	68.7
20	426	180	62.4
22	391	200	57.4
24	361	250	49.1
26	336	300	43.2
28	314	350	38.8
30	296	400	35.4
35	257	500	30.9
40	228	600	27.7
45	215	800	23.4
50	187	1000	20.9
55	171	1500	17.1
60	158	2000	14.8
65	147	3000	12.1

Procedure.—Push the rod down into the water vertically as far as the wire can be seen and then read the level of the surface of the water on the graduated scale. This will indicate the turbidity.

The following precautions shall be taken to insure correct results:

Observations shall be made in the open air, preferably in the middle of the day and not in direct sunlight. The wire shall be kept bright and clean. If for any reason observations cannot be made directly under natural conditions, a pail or tank may be filled with water and the observation taken in that, but in this case care shall be taken that the water is thoroughly stirred before the observation is made, and no vessel shall be used for this purpose unless its

diameter is at least twice as great as the depth to which the wire is immersed. Waters which have a turbidity above 500 shall be diluted with clear water before the observations are made, but in case this is done the degree of dilution used shall be stated and form a part of the report.

The wire method shall be used for testing the degree of fineness of the standard silica, and this degree of fineness shall be such that when added to distilled water in an amount equal to 100 parts per million, the wire observed under standard conditions can be just seen at a depth of 100 mm. below the surface of the water.

TURBIDIMETRIC METHOD.

Several forms of turbidimeters (or diaphanometers¹¹) have been suggested for use, but as improvements in them are being constantly made no definite form is here prescribed for use. The simplest and most satisfactory form at present is the candle turbidimeter.¹² *

This consists of a graduated glass tube with a flat polished bottom, enclosed in a metal case. This is held over an English standard candle and so arranged that one may look vertically down through the tube and see the image of the candle. The observation is made by pouring the sample of water into the tube until the image of the candle just disappears from view. Care shall be taken not to allow soot or moisture to accumulate on the lower side of the glass bottom of the tube so as to interfere with the accuracy of the observations. The graduations on the tube correspond to turbidities produced in distilled water by certain numbers of parts per million of silica standard. In order to insure uniform results it is necessary to have the distance between the top rim of the candle and the bottom of the tube constant, and this distance shall be three inches, or 7.6 cm. The observations shall be made in a darkened room or with a black cloth over the head.

All apparatus of this description shall be calibrated to correspond with the United States Geological Survey scale. The following figures are believed to be approximately correct for the candle turbidimeter, but should be checked by the experimenter.

* Manufactured by Baker and Fox, 83 Schermerhorn St., Brooklyn, N. Y.

TABLE 3.

Depth in Centimeters	Turbidity Parts per Million of Silica	Depth in Centimeters	Turbidity Parts per Million of Silica
2.3	1000	7.5	290
2.6	900	7.8	280
2.9	800	8.1	270
3.2	700	8.4	260
3.5	650	8.7	250
3.8	600	9.1	240
4.1	550	9.5	230
4.5	500	9.9	220
4.9	450	10.3	210
5.5	400	10.9	200
5.6	390	11.4	190
5.8	380	12.0	180
5.9	370	12.7	170
6.1	360	13.5	160
6.3	350	14.4	150
6.4	340	15.4	140
6.6	330	16.6	130
6.8	320	18.0	120
7.0	310	19.6	110
7.3	300	21.5	100

EXPRESSION OF RESULTS.

The results of turbidity observations shall be expressed in whole numbers which correspond to parts per million of silica, and recorded as follows:

Turbidity between	1 and	50	Recorded to nearest unit
"	"	51 "	100 " " " 5
"	"	101 "	500 " " " 10
"	"	501 "	1000 " " " 50
"	"	1001 "	above " " " 100

COLOR.

The "color" of water, or the "true color," shall be considered as that part of the apparent color which is due only to substances in solution; that is, it is the color of the water after the suspended matter has been removed.

The "apparent color" shall be considered as including not only the true color but also any color produced by substances in suspension. It is the color as viewed by inspection of the original sample.

In stating the results the word "color" shall mean the "true color" unless otherwise expressed.

The platinum-cobalt method of measuring color shall be con-

sidered as the standard, and the unit of color shall be that produced by one part per million of platinum.

PLATINUM-COBALT STANDARD.¹³

The standard solution, which has a color of 500, shall be prepared as follows:

Dissolve 1.246 grams of potassium platinic chloride ($\text{PtCl}_4 \cdot 2\text{KCl}$) containing 0.5 gram platinum, and one gram crystallized cobalt chloride ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) containing 0.25 gram of cobalt in water, with 100 c.c. concentrated hydrochloric acid, and make up to one liter with distilled water.

By diluting this solution with distilled water to the 100 c.c. graduation mark on the nessler tubes, standards shall be prepared having colors of 0, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60 and 70. These shall be kept in nessler tubes of such diameter that the 100 c.c. graduation mark is between 20 and 25 cm. above the bottom and is uniform for all tubes. They shall be protected from dust when not in use.

Procedure.—The color of a sample shall be observed by filling a standard nessler tube to the graduation mark with the water to be examined to a depth equal to that of the standards and by comparing it with the standards. The observation shall be made by looking vertically downwards through the tubes upon a white surface placed at such an angle that light is reflected upwards through the column of liquid.

Waters that have a color darker than 70 shall be diluted before making the comparison, in order that no difficulties may be encountered in matching the hues.

Water containing matter in suspension shall be filtered, before the color observation is made, until no visible turbidity remains. If the suspended matter is coarse, filter paper may be used for this purpose; if the suspended matter is fine, the use of a Berkefeld filter is recommended. The Pasteur filter shall not be used, as it exerts a marked decolorizing action.

The apparent color shall be determined on the original sample without filtration. In the case of clear waters or waters with low turbidities, the true color and the apparent color are substantially the same.

EXPRESSION OF RESULTS.

The results of color determination shall be expressed in whole numbers and not in hundredths, as was formerly the case, and recorded as follow:

Color between	1	and	50	Recorded to nearest unit
"	"	51	" 100	" " " 5
"	"	101	" 250	" " " 10
"	"	251	" 500	" " " 20

U. S. GEOLOGICAL SURVEY FIELD METHOD.¹⁰

As the above described method is not well adapted for field work, a method has been devised by which the color of the water to be tested may be compared with that of glass disks* held at the end of metallic tubes through which they are viewed by looking towards a white surface. When this method is used the glass disks are individually calibrated (by the makers*) to correspond with colors on the platinum scale. Experience has shown that the glass disk method used by the U. S. Geological Survey gives results in substantial agreement with those obtained by the platinum determinations, and its use is recognized as a standard procedure.

COMPARISON WITH NESSLER STANDARDS.

Inasmuch as the nessler scale¹⁴ (and the natural water scale,¹⁵ which agrees with it except for the figures below 20) has been largely used in the past, it is often desirable to transpose¹⁶ the old results to the platinum standard. For this purpose the ratios given in Table 4 may be conveniently employed, but they must not be considered as universally applicable, as the character of the nessler solution introduces an uncertain factor.

RECORDS OF TINTS AND SHADES OF APPARENT COLOR.

The value of the readings of tint and shade by the Lovibond Tintometer¹⁷ has not been commensurate with the labor involved, but it is necessary in some cases to make a record of the reflected tint and shade¹⁸ of the water.

The standard color disks† used in teaching optics may be used for the purpose.

*This apparatus is made by the Builders Iron Foundry, Providence, R. I.

†Manufactured by the Milton Bradley Educational Co., Springfield, Mass.

TABLE 4.

TABLE FOR CONVERTING COLORS BY THE NATURAL WATER SCALE INTO PARTS PER MILLION OF COLOR BY THE PLATINUM STANDARD.*

Modified Nessler or Natural-Water Standard	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
0.00	0	2	4	6	8	9	11	13	15	17
0.10	18	19	20	20	21	22	23	24	24	26
0.20	26	27	27	28	29	29	30	31	32	32
0.30	33	34	34	35	35	36	37	37	38	38
0.40	39	40	40	41	42	42	43	44	45	45
0.50	46	47	47	48	48	49	50	50	51	51
0.60	52	53	53	54	54	55	56	56	57	57
0.70	58	58	59	59	60	60	61	61	62	62
0.80	63	64	64	65	66	66	67	68	69	69
0.90	70	71	72	73	74	75	77	78	79	80
1.00	81	82	82	83	84	85	86	86	87	87
1.10	88	89	89	90	91	91	92	93	94	94
1.20	95	96	96	97	98	98	99	100	101	101
1.30	102	103	103	104	105	105	106	107	108	108
1.40	109	110	110	111	112	112	113	114	115	115
1.50	116	117	117	118	118	119	120	120	121	121
1.60	122	123	123	124	125	125	126	127	128	128
1.70	129	130	130	131	132	132	133	134	135	136
1.80	136	137	137	138	139	139	140	141	142	142
1.90	143	144	144	145	146	146	147	148	149	149
2.00	150

Procedure.—The white disk supports three movable standard color sectors, red, yellow and blue, also one movable black sector. The whole is mounted on a device which can be revolved rapidly and which blends the colors into a uniform tint or shade. Around the circumference of the disk there is a scale which is used to indicate the percentage of each color or white or black in the blend.

Place the sample in a battery jar on a white background; adjust the sectors so that when blended the tint or shade will match the reflected tint or shade of the sample. Express the results as the percentages of red, yellow, blue, white and black in the blended tint or shade.

ODOR.¹⁰

The observation of the odor of cold and hot samples of surface waters is very important, as the odors are usually connected with some organic growths or with sewage contamination, or both.

The odor of ground waters is often caused by the earthy constituents of the water-bearing strata. The odor of a contaminated well water is often decisive evidence of its pollution.

*The zero on the true Nessler scale reads about 15 on the platinum scale.

A study of the organisms as directed under Microscopical Examination, page 80, is an invaluable adjunct to the physical and chemical examination of water. Certain organisms can be distinguished by their odor, as, for example, the "fishy" odor of *Uroglena*, the "aromatic" or "rose geranium" odor of *Asterionella* and the "pig-pen" odor of *Anabaena*.

Procedure.—Observe and record the odor, both at room temperature and at just below the boiling-point, as follows:

Cold Odor.—Shake the sample violently in one of the gallon collecting bottles, when it is from about half or two-thirds full and when the sample is at room temperature (about 20° C.). Remove the stopper and smell the odor at the mouth of the bottle.

Hot Odor.—Into a tall 400 c.c. beaker without lip pour about 150 c.c. of the sample. Cover the beaker with a well-fitting watch glass, place on a hot plate and bring the water to just below boiling. Remove the beaker from the plate and allow it to cool for not more than five minutes. Then shake with a rotary movement, slip the watch glass to one side and smell the odor.

EXPRESSION OF RESULTS.

Express the quality of the odor by some such descriptive epithet as the following, which for purposes of record may be abbreviated:

v—vegetable.	m—moldy.
a—aromatic.	M—musty.
g—grassy.	d—disagreeable.
f—fishy.	p—peaty.
e—earthy.	s—sweetish.

Express the intensity of the odor by a numeral prefixed to the term expressing quality, which may be defined as follows:

Numerical Value.	Term.	Approximate Definition.
0	None.	No odor perceptible.
1	Very faint.	An odor that would not be ordinarily detected by the average consumer, but that could be detected in the laboratory by an experienced observer.
2	Faint.	An odor that the consumer might detect if his attention were called to it, but that would not otherwise attract attention.

Numerical Value.	Term.	Approximate Definition.
3	Distinct.	An odor that would be readily detected and that might cause the water to be regarded with disfavor.
4	Decided.	An odor that would force itself upon the attention and that might make the water unpalatable.
5	Very strong.	An odor of such intensity that the water would be absolutely unfit to drink. (A term to be used only in extreme cases.)

CHEMICAL EXAMINATION.

The scope of the chemical determinations considered in this report is sufficiently indicated by the table of contents, and the general applicability of each determination according to the views of the committee is set forth in the letter of transmittal. No further introduction is required except to refer to the brief description of the nature and purpose of each determination which precedes the various methods given.

EXPRESSION OF RESULTS.

The committee recommends that the results of chemical analyses be expressed in parts per million, which in most cases is practically equivalent to milligrams per liter. Nevertheless it is recognized that in some of the older laboratories, where for many years other forms of expression have been used, it may not be expedient to change their present practice at once.

The results expressed in parts per 100,000 or in grains per gallon may be transformed to parts per million, or conversely, by the use of the following table:

TABLE 5.

	Grains per U. S. Gallon	Grains per Imperial Gallon	Parts per 100,000	Parts per 1,000,000
1 grain per U. S. gallon	1.000	1.20	1.71	17.1
1 grain per Imperial gallon	0.835	1.00	1.43	14.3
1 part per 100,000	0.535	0.70	1.00	10.0
1 part per 1,000,000	0.053	0.07	0.10	1.0

The committee desires to call attention to the practice of many analysts of using too many decimals in expressing the results of

analyses. In some instances the records imply that the methods of sampling and analysis are 10 or even 100 times more accurate than the facts warrant. The following general rules are advised:

1. When the results show quantities above 10 parts per million, do not use any decimals; record only whole numbers. In fact, where the quantities reach hundreds and thousands of parts, there is much merit in recording a cipher in the units place, and in following the general manner of expression given under Turbidity, page 20.

2. When the results are between 1 and 10 parts, do not carry decimals to more than one place.

3. When the results are between 0.1 and 1 part, do not carry decimals to more than two places.

4. Nitrogen in drinking waters as free and albuminoid ammonias, and as nitrite, alone justify the use of three decimals.

Where there are tabulated together the results of various analyses calling for two or more of the above rules to be applied in the same column, the committee suggests that if desired ciphers and not digits be used at the right of the larger figures to make the column uniform in appearance.

DETERMINATION OF OXYGEN CONSUMED.²⁰

"Oxygen consumed" means the oxygen which the organic compounds of sewage and waters consume when treated in an acid solution with potassium permanganate. The expression is synonymous with "oxygen required" and with "oxygen absorbed." The expression "dissolved oxygen" refers to another determination.

It is the carbon, and not the nitrogen, in organic matter which is oxidized in this way by potassium permanganate, hence this determination is frequently referred to as an indication of the carbonaceous organic matter present. However, it indicates only a certain portion of the carbon, and this ratio varies in different samples of water and of sewage. Furthermore, it does not differentiate the carbon present in unstable organic matter from that in what might be called fairly stable organic matter, such as is sometimes referred to as residual humus matter.

If nitrites, ferrous iron, sulphides or other unoxidized mineral compounds are present they will increase the oxygen consumed; and hence a correction should be made for them when studying carbonaceous organic matter.

It is one of the oldest methods for determining organic matter, and in fact has been in very wide use for more than half a century. Its introduction followed the recognition of the fact that the loss on ignition of the residue upon evaporation may indicate certain volatile mineral matters as well as organic matter. Unfortunately this determination has been made by a great variety of procedures as to certain details, and these variations have been detrimental to establishing the method on the most favorable basis.

The essential features of the method common to all the various procedures will be first described, after which will be taken up the variable factors, namely, the temperature and time that the permanganate solution is allowed to act on the sample under examination.

Reagents.—1. Dilute sulphuric acid. One part of sulphuric acid to three parts of distilled water. This shall be freed from oxidizable matters by adding potassium permanganate until a faint pink color persists after standing several hours.

2. Standard potassium permanganate solution. Dissolve 0.4 gram of the crystalline compound in one liter of distilled water. Standardize against an ammonium oxalate solution. One c.c. is equivalent to 0.0001 gram of available oxygen.

3. Ammonium oxalate solution. Dissolve 0.888 gram of the substance in one liter of distilled water. One c.c. is equivalent to 0.0001 gram of oxygen.

4. Potassium iodide solution. Ten per cent solution free of iodate.

5. Sodium thiosulphate solution. Dissolve 1.0 gram of the pure crystallized salt in one liter of distilled water. Standardize against a potassium permanganate solution which has been standardized against an ammonium oxalate solution. As this solution does not keep well, determine its actual strength at frequent intervals.

6. Starch indicator. Prepare as directed beyond under Dissolved Oxygen, page 74.

Procedure.—Measure into a flask 100 c.c. of the water, or a smaller diluted portion if the water is of high organic content. Add 10 c.c. of sulphuric acid solution and 10 c.c. of potassium permanganate solution, and allow the treated sample of water to digest in accordance with the detailed procedures carried out under differing conditions as set forth below under the heading Period and Temperature of Digestion, page 29.

Precisely at the end of the period of digestion, remove the flask, and if the boiling temperature is used, add 10 c.c. of the ammonium oxalate solution. Titrate with the permanganate solution until a faint but distinct color is obtained.

Each c.c. of the permanganate solution in excess of the oxalate solution represents 0.0001 gram of oxygen consumed by the sample.

At the end of the period of digestion, if not made at the boiling temperature, add 0.5 c.c. of potassium iodide solution to discharge the pink color; mix; titrate the liberated iodine with thiosulphate until the yellow color is nearly destroyed, then add a few drops of starch solution and continue titration until the blue color is just discharged.

Should the volume of permanganate solution be insufficient for complete oxidation, repeat the analysis, using a larger volume so that at least three c.c. of the permanganate solution will be present in excess when the ammonium oxalate solution is added.

When unoxidized mineral substances, such as ferrous sulphate, sulphides, nitrites, etc., are present in the sample, corrections should be applied as accurately as possible by procedures suitable for the samples being analyzed. Direct titration of the acidified sample in the cold, using a three minute period of digestion, serves this purpose quite well for polluted surface waters and fairly well for purified sewage effluents. Raw sewages containing no trade wastes seldom need such a correction; but when raw sewages contain "pickling liquors" it is important. In all samples containing both unoxidized mineral compounds and gaseous organic substances, the latter should be driven off by heat and the sample

allowed to cool before applying this test for the correction factor. Where such corrections are necessary the fact should be stated, with the amount of correction.

Period and Temperature of Digestion.—Unfortunately, widely varying details are practiced in this regard. This means that it is difficult to compare the results obtained at one laboratory with those obtained at another; and, furthermore, the amount of data obtained at various places is so great that it is awkward and difficult to make a change at some laboratories, as future data obtained by modified methods will not be directly comparable with existing data obtained by the present methods. None of the methods gives absolute results; they are only relative²¹ at best. The principal methods may be mentioned as follows:

1. Bring the acidified sample to the boiling point, add the permanganate solution and digest for two minutes²² at a boiling temperature. This procedure is facilitated by agitating the liquid constantly with a small current of air to guard against bumping.

2. Same method as No. 1 except that the period of digestion is five minutes²³ instead of two minutes.

3. Same method as No. 2 except that the permanganate solution is added to the acidulated sample when cold, and the period of digestion continued for five minutes after the sample reaches the boiling point. The advantage of this method is that there are recorded the oxygen consuming powers of the volatile matters present in some sewages and sewage effluents, which are driven off by heat and thus escape when analyses are made in accordance with the first two procedures above noted.

4. Same method as No. 3 except that the period of digestion is 10 minutes²⁴ instead of five minutes.

5. The permanganate is added in the cold to the acidulated sample and the flask placed immediately in a bath of boiling water, the water level of which is kept above the level of the contents in the flask. Digestion is continued for exactly 30 minutes.²⁵

6. Digestion of the sample after the acid and permanganate solutions are added is carried out abroad, especially in England, at approximately the room temperature,²⁶ apparently to guard against decomposition²⁷ of permanganate in the presence of high

chlorine, for periods of three minutes, 15 minutes and four hours; many observers record the oxygen consumed after all three periods, while some record the result only for the four-hour period.

Concluding Notes.—After careful consideration of the matter the views of the committee are as follows:

1. From a strictly scientific standpoint the 30-minute period of digestion at boiling temperature in a water bath appears to give the most satisfactory results as regards uniformity and freedom from personal errors of manipulation. It is believed that where practicable this is the most satisfactory method for adoption.

2. Where samples from a given source are repeatedly analyzed it is advised that there be placed on record, for purposes of comparison, representative results by each of the methods of digestion above mentioned.

3. In connection with sewage works analysis, to which the usefulness of this method is principally confined (see Table 1), it is recommended that the permanganate solution be added to the sample before heating in order to include the oxygen consumed by volatile compounds.

DETERMINATION OF NITROGEN.

Nitrogenous organic matter, due to natural agencies, passes from crude organic matter through several intermediate compounds and (that which does not gasify) ultimately forms nitrates.

Nitrogen in the form of organic matter can be determined as organic nitrogen by the so-called Kjeldahl process,²⁸ and it can be also approximated by the albuminoid ammonia determination.²⁹ It is not possible to differentiate the nitrogen in the organic matter which readily decomposes from that in stable or non-putrescible compounds.

Decomposition of organic matter produces nitrogen in the form of free or saline ammonia, which is the first intermediate step between crude nitrogenous organic matter and the completely mineralized matter in the form of nitrates. Nitrogen as free ammonia may be determined by distillation and nesslerization, or by the direct nesslerization of the clarified sample.

The second intermediate form in which nitrogen occurs is that of nitrites.

When nitrogenous organic matter is completely mineralized, as above stated, it is present in the form of nitrates.

The importance of the determination of nitrogen in working with sewage and unpurified water supplies causes this set of determinations to be of much significance. They are described at length, from the standpoint of regular laboratory practice, supplemented in some particulars from the standpoint of field work such as is required in the operation of sewage works.

NITROGEN AS FREE AMMONIA.

There are two methods for estimating nitrogen as free ammonia, namely, (A) by distillation, and (B) by direct nesslerization.

The former is recommended for general use, although for sewages, sewage effluents and highly polluted surface waters the great convenience and apparent adequacy of the latter are fully recognized. It is further believed that the slight loss in ammonia by direct nesslerization, perhaps 10 per cent on an average, is probably no more serious and perhaps less so than the inclusion by distillation of intermediate compounds, which do not strictly belong to nitrogen as free ammonia. The difficulty, however, of obtaining a proper treatment of some samples so that they will not become cloudy when nesslerized, makes this procedure, in the opinion of some, rather too uncertain for general adoption at this time. Nevertheless, it is the opinion of the committee that more thorough study will cause the apparent objection to direct nesslerization to disappear, and that wider familiarity with this method will soon bring it into general use for sewage work, as is now the case to some degree abroad³⁰ and at a few places in this country.

For waters low in free ammonia it is now somewhat uncertain what will be the future of the direct nesslerization method.

*Free Ammonia by Distillation.*³¹

Procedure.—A metal or glass flask, connected to the condenser³² in such a way that the distillate may be conveniently delivered from the tin or aluminum condenser tubes directly into the nessler tubes, shall be freed from ammonia by boiling distilled water in it, until the distillate shows no further traces of free ammonia. When this has been done, empty the distilling flask

and measure into it 500 c.c. of the sample, or a smaller portion diluted to 500 c.c. Apply heat so that the distillation will be at the rate of not more than 10 c.c. nor less than 6 c.c. per minute.

Collect three nessler tubes of the distillate, 50 c.c. to each portion; these contain the free ammonia to be measured as described below.

If the sample is acid, or if the presence of urea is suspected, add about 0.5 gram of sodium carbonate previous to distillation. Omit this when possible, as it tends to increase "bumping."

Use only nessler tubes which do not show a variation of more than six mm. (0.25 inch) in the distance which the graduation mark (50 c.c.) is above the bottom. The tubes shall be of clear white glass, with polished bottoms.

Measurement of Nitrogen as Ammonia.

This measurement may be made either by (1) comparison of the nesslerized distillates with nesslerized solutions containing known quantities of nitrogen as ammonium chloride, or by (2) comparison of the nesslerized distillates with permanent standard solutions in which the colors of nesslerized standard ammonia solutions are duplicated by solutions of platinum and cobalt chlorides.

Comparison with Ammonia Standards.

Reagents.—1. Ammonia-free water.

2. Standard ammonium chloride solution. Dissolve 3.82 grams of ammonium chloride in one liter of distilled water; dilute 10 c.c. of this to one liter with ammonia-free water. One c.c. equals 0.00001 gram of nitrogen.

3. Nessler's reagent. Dissolve 50 grams potassium iodide in a minimum quantity of cold water. Add a saturated solution of mercuric chloride until a slight but permanent precipitate persists. Add 400 c.c. of 50 per cent solution of potassium hydrate, made by dissolving the potassium hydrate and allowing it to clarify by sedimentation before using. Dilute to one liter, allow to settle and decant.³³ This solution should give the required color with ammonia within five minutes after addition, and should not precipitate with small amounts of ammonia within two hours.

Prepare a series of 16 Nessler tubes which contain the following numbers of c.c. of the standard ammonium chloride solution, diluted to 50 c.c. with ammonia-free water, namely: 0.0, 0.1, 0.3, 0.5, 0.7, 1.0, 1.4, 1.7, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 6.0. These will contain 0.00001 gram of nitrogen for each c.c. of the standard solution used.

Nesslerize the standards and also the distillates by adding approximately two c.c. of nessler reagent to each tube. Do not stir the contents of the tubes.

Have the temperature³⁴ of the tubes practically the same as that of the standards, otherwise the colors will not be directly comparable.

Compare the color produced in these tubes with that in the standards by looking vertically downward through them at a white surface placed at an angle in front of a window so as to reflect the light upwards. Allow the tubes to stand for at least 10 minutes after nesslerizing before making the comparison.

In case the color obtained by nesslerizing the distillates is greater than that of the darkest tube of the standards, mix the contents of the tube thoroughly and pour out half of the liquid, making up the remainder to the original volume with ammonia-free water, then make the color comparison and multiply the result by two. If, after pouring out half of the liquid, the color is still too dark, repeat this process of division until a reading can be made.

In case the color of the distillates is too high, this process may be shortened by mixing together all of the distillates from one sample before making the comparison, subsequently taking an aliquot portion for comparing with the standards.

After the readings have been made and recorded, add together the results obtained by nesslerizing each portion of the entire distillate from each sample. If 500 c.c. of the sample are distilled, this sum multiplied by .02 will give the number of parts per million of nitrogen as free ammonia in the sample.

Comparison with Permanent Standards.³⁵

In this method, which is rapidly coming into general use in the large laboratories, the distillates are nesslerized in the same

manner as above described; and the resulting colors at the end of about 10 minutes are compared with permanent standards made as follows:

Platinum Solution.—Weigh out two grams of potassium platonic chloride ($\text{Pt Cl}_4 2\text{KCl}$), dissolve in a small amount of distilled water, add 100 c.c. of strong hydrochloric acid and make up to one liter.

Cobalt Solution.—Weigh out 12 grams of cobaltous chloride ($\text{CoCl}_2 6\text{H}_2\text{O}$), dissolve in distilled water; add 100 c.c. of strong hydrochloric acid and make up to one liter.

Prepare standards by putting varying amounts of these two solutions in nessler tubes filling up to the 50 c.c. mark with distilled water as follows:

TABLE 6.

Equivalent Volume of Standard Ammonium Chloride c.c.	Platinum Solution c.c.	Cobalt Solution c.c.
0.0	1.2	0.0
0.1	1.8	0.0
0.3	2.8	0.0
0.5	4.7	0.1
0.7	5.9	0.2
1.0	7.7	0.5
1.4	9.9	1.1
1.7	11.4	1.7
2.0	12.7	2.2
2.5	15.0	3.3
3.0	17.3	4.5
3.5	19.0	5.7
4.0	19.7	7.1
4.5	19.9	8.7
5.0	20.0	10.4
6.0	20.0	15.0
7.0	20.0	22.0

It is necessary to use tubes which have the 50 c.c. mark not less than 20 nor more than 22 cm. above the bottom.

These standards may be kept for several months if protected from dust.

The method of calculating results is precisely the same as with the ammonia standards.

Modification of the Distillation Process for Sewages.

The determination of free and albuminoid ammonia in sewages, soils and other material of high nitrogen content may be satis-

factorily made by first diluting the sample with ammonia-free distilled water, and proceeding as above described; but it is generally preferable, especially in connection with albuminoid ammonia tests, to use the steam method,* as follows:

Procedure.—Put the sample to be tested, after the entire apparatus is freed from ammonia, in a long Kjeldahl flask which has a capacity of about 200 c.c., using such an amount of the sample that the color of the nesslerized distillates will fall within the range of the standards.

Generate ammonia-free steam in an ordinary distilling flask and pass it through the liquid in the Kjeldahl flask by means of a glass tube which extends almost to the bottom of the latter. Connect the neck of the Kjeldahl flask with the condenser in the usual way.

This method has the advantage of yielding the free ammonia and also the albuminoid ammonia, when the sample is treated with an alkaline solution of permanganate, more promptly than by the ordinary process of distillation; and, furthermore, “bumping” is avoided. It also permits the assay of solid matter.

It is often convenient to collect the free ammonia distillate in a single receptacle and to take an aliquot part of it for nesslerization.

*Free Ammonia by Direct Nesslerization.**

Reagents.—1. A 10 per cent solution of copper sulphate.

2. A 10 per cent solution of lead acetate.

3. A 50 per cent solution of sodium or potassium hydrate.

4. A 10 per cent solution of magnesium chloride.

*Procedure (1) for Sewage.**—Fifty c.c. of the sample to be tested are mixed with an equal volume of water, placed in a short nessler tube and a few drops of copper sulphate solution added. After a thorough mixing, one c.c. of the potassium hydrate solution is added and the contents are again thoroughly mixed. The tube is then allowed to stand for a few moments, when a heavy precipitate should fall to the bottom, leaving a colorless supernatant liquid. Nesslerize an aliquot portion of this clear liquid.

*Procedure (2) for Sewage.*³⁹—In place of adding copper sulphate to sewages of high magnesium content, it has been found that satisfactory clarification and also softening of the sample may be obtained by heating it to 40° C. after mixing with the caustic alkali. The heat causes the bicarbonate of lime to be precipitated and the magnesium to separate as a gelatinous precipitate (hydrate). During cooling, the bottle containing 100 c.c. of the sample should be shaken several times to facilitate the subsidence of the precipitate. Where samples are low in magnesium content this treatment may be accomplished by adding a small quantity of magnesium chloride.

Many samples containing hydrogen sulphide require the use of lead acetate in addition to the copper, and others require a few trials before the right combination of the three solutions to bring about the best results can be made. In view of the fact that flocculent precipitates absorb varying amounts of ammonia from solution under certain conditions, it is recommended that the smallest practicable amounts of precipitants be used.

The amount of nitrogen as free ammonia is computed after comparisons with standards in the same manner as in the distillation procedure.

NITROGEN AS ALBUMINOID AMMONIA.

The addition of an alkaline permanganate solution to liquids containing nitrogenous organic matter causes the formation of ammonia, the amount of which can be measured upon distillation of the treated sample and the nesslerization of the distillate. In sewages and other liquids and substances containing considerable nitrogenous organic matter the percentage of ammonia-forming organic matter is decidedly variable.⁴⁰ For this reason albuminoid ammonia results in such cases are less valuable⁴¹ than the total organic nitrogen, called by some the Kjeldahl nitrogen. Hence for sewage work, including the analyses of both the influents and effluents of purification plants, as well as the study of highly polluted streams, it is recommended that albuminoid ammonia determinations be omitted and in their place the total organic nitrogen be determined.

For ground waters and surface waters containing but little

pollution, the nitrogen as albuminoid ammonia quite uniformly approximates about one-half of the total organic nitrogen. Accordingly the continuance of albuminoid ammonia determinations for this class of work is approved. Nevertheless the inferiority of such results to those of total organic nitrogen is recognized.

The method shall be applied in conjunction with that for free ammonia, as follows:

Reagents.—Alkaline potassium permanganate. Pour 1200 c.c. of distilled water into a porcelain dish holding 2500 c.c., boil 10 minutes and turn off the gas. Add 16 grams of C. P. potassium permanganate and stir until dissolved. Then add 800 c.c. of 50 per cent clarified solution of potassium or sodium hydrate and enough distilled water to fill the dish. Boil down to 2000 c.c. Test each batch of this solution for albuminoid ammonia by making a blank determination. Correction should be made accordingly.

Procedure.—Interrupt the distillation (made as already described) after the collection of the distillate for free ammonia.

Add 40 c.c. or more of alkaline potassium permanganate and conduct this distillation until at least four portions of 50 c.c. each and preferably five portions of the distillate have been collected in separate tubes.

Have enough permanganate solution present to insure the maximum oxidation of the organic matter. These distillates contain the nitrogen as albuminoid ammonia, measurement of which shall be made as described in connection with nitrogen as free ammonia.

Dissolved nitrogen as albuminoid ammonia may be determined from a sample from which suspended matters are removed by filtration either through filter paper, or, if finely divided matters are present, through a Berkefeld filter.

Suspended nitrogen as albuminoid ammonia may be obtained by taking the difference between the total and dissolved results.

The results shall be expressed as in the case of free ammonia.

TOTAL ORGANIC NITROGEN.⁴²

The total organic nitrogen, the significance of which has been already described, shall be determined as follows:

Boil 500 c.c. of the sample in a round bottom flask until free of ammonia. This usually requires the loss of about 200 c.c. of the sample, which, if desired, may be collected for the determination of free ammonia.

Add five c.c. of C. P. concentrated sulphuric acid which is free of nitrogen, together with a small piece of ignited pumice. Mix by shaking and place over a flame under a hood.

Digest until copious fumes of sulphuric acid are given off and until the liquid chars and finally becomes colorless. Remove from the flame, add potassium permanganate in small portions until a heavy green precipitate persists in the liquid. Cool. Dilute with about 100 c.c. of ammonia-free water. Neutralize with ammonia-free sodium carbonate solution (10 per cent). Distill off the ammonia, collect in nessler tubes and nesslerize and compare with standards as already described.

In the case of sewage it is preferable to distill the free ammonia, by passing live steam through 100 c.c. or less of the sample. Add five c.c. of sulphuric acid and digest, dilute the colorless digested liquid to 500 c.c. Place 10 c.c. or more of this liquid in a 200 c.c. Kjeldahl distilling flask. Dilute with 100 c.c. of water. Neutralize with 10 c.c. of sodium carbonate solution (10 per cent solution), distill with steam and nesslerize.

In this determination care must be taken to digest thoroughly, to add potassium permanganate to the point of precipitation, to sample carefully after dilution and to add enough sodium carbonate to insure the separation of the ammonia from the precipitated manganese hydrate. Potassium permanganate shall not be added during digestion because it causes loss of nitrogen.

NITROGEN AS NITRITES.⁴³

The following shall be considered the standard mode of procedure for water and sewages in determining the nitrogen as nitrites, the second intermediate step by which nitrogenous matter passes from crude organic matter to mineral matter (nitrates). Nitrites may also be formed by the reduction of nitrates.

Reagents.—1. Sulphanilic acid solution. Dissolve eight grams of the purest sulphanilic acid in 1000 c.c. of 5 N. acetic acid (Sp. Gr. 1.041). This is a practically saturated solution.

2. *a*-amidonaphthalene acetate solution. Dissolve 5.0 grams solid *a*-naphthylamine in 1000 c.c. of 5 N. acetic acid; filter the solution through washed absorbent cotton.

3. Sodium nitrite, stock solution. Dissolve 1.1 gram silver nitrite in nitrite-free water; precipitate the silver with sodium chloride solution and dilute the whole to one liter.

4. Standard sodium nitrite solution. Dilute 100 c.c. of solution (3) to one liter; then dilute 10 c.c. of this solution to one liter with sterilized nitrite-free water; add one c.c. of chloroform and preserve in a sterilized bottle. One c.c. = 0.0000001 gram nitrogen.

Procedure.—Measure out 100 c.c. of the decolorized sample (decolorized by adding aluminum hydrate free of nitrite—see under Chlorine), or a smaller portion diluted to 100 c.c., into a nessler tube. These nessler tubes shall be of clear white glass, with the 100 c.c. graduation mark not varying more than six mm. in its distance above the bottom. At the same time make a set of standards by diluting various volumes of the standard nitrite solution in Nessler tubes to 100 c.c. with nitrite-free water, for example, 0, 1, 3, 5, 7, 10, 14, 17, 20 and 25 c.c. Add two c.c. of reagents Nos. 1 and 2 (above) to each 100 c.c. of the sample and to each standard. Mix, allow to stand 10 minutes. Compare the samples with the standards. Do not allow the samples to stand over one-half hour before being compared. Make a blank determination in all cases to correct for the presence of nitrite in the air, the water and the reagents. Dilute all samples which develop more color than the 30 c.c. standard before comparing. Mixing is important.

The solution must be acid. Hydrochloric acid, which until recently was in quite general use in this country for a solvent for the naphthalene, permits satisfactory results to be obtained, but the speed of the reaction is much slower than in the case of acetic acid." For this reason the latter acid is preferred.

When 100 c.c. of the sample are used, then 0.001 times the number of c.c. of the standard gives the parts per million of nitrogen as nitrite.

NITROGEN AS NITRATES.⁴⁵

No single method appears to be applicable to the determination of nitrogen as nitrates in all classes of water, sewages and sewage effluents, and there is no method which is not subject to considerable error.

Where the amount of chlorine in the sample is less than about 30 parts per million, the phenolsulphonic acid method is recommended. When the chlorine is greater than this, as in sewage work, the reduction method is recommended.

The standard mode of procedure for these methods shall be as follows:

*Phenolsulphonic Acid Method for Nitrates.*⁴⁶

Reagents.—1. Phenolsulphonic acid. Mix 30 grams of synthetic phenol with 370 grams of C. P. concentrated sulphuric acid in a round-bottom flask. Put this flask in a water bath and support it in such a way that it shall be completely immersed in the water. Heat for six hours.

2. Ammonium hydrate solution diluted with distilled water 1 to 1. Potassium hydrate may be used.

3. Standard nitrate solution. Dissolve 0.72 gram of pure recrystallized potassium nitrate in one liter of distilled water. Evaporate cautiously 10 c.c. of this strong solution on the water bath. Moisten quickly and thoroughly with two c.c. of phenolsulphonic acid and dilute to one liter for the standard solution, one c.c. of which equals .000001 gram of nitrogen.

Procedure—Evaporate 20 c.c. or less of the sample in a small porcelain evaporating dish on the water bath, removing it from the bath just before it has come to dryness. Let the last few drops evaporate at room temperature in a place protected from the dust. When the sample is suspected to contain a large amount of nitrate, evaporate less than 20 c.c. If it is suspected to contain but little, evaporate more.

If the sample has a high color, decolorize before evaporating by the use of washed aluminum hydrate, as directed in connection with the chlorine determination.

Add one c.c. of phenolsulphonic acid and rub this quickly and thoroughly over the residue with a glass rod. Add about

10 c.c. of distilled water and stir with a glass rod until mixed. Add enough ammonium hydrate solution (or potassium hydrate if the operation must of necessity be carried on in a room where ammonia distillations are made) to render the liquid alkaline. Transfer the liquid to a 100 c.c. nessler tube and fill the tube to the 100 c.c. mark with distilled water.

If nitrates are present there will be formed a yellow color; this may be compared with permanent standards⁴⁶ made for the purpose, and kept satisfactorily for several weeks. The series of standards for comparison shall be made by putting the following quantities of the standard solution into 100 c.c. tubes and making up to the 100 c.c. mark with distilled water, adding five c.c. of strong ammonia to each tube :

TABLE 7.

Amount of Dilute Standard Added	Standard Nitrate
c.c.	milligram
0	0.000
1.0	0.001
3.0	0.003
5.0	0.005
7.0	0.007
10.0	0.010
15.0	0.015
20.0	0.020
25.0	0.025
30.0	0.030
35.0	0.035
40.0	0.040

Compare the sample treated as above described with these standards by looking down vertically through the tubes at a white surface so placed in front of a window that it will reflect the light upward through them.

If the figures obtained by this comparison be divided by the number of c.c. of the samples which were evaporated, the quotient gives the number of parts per million of nitrogen in the form of nitrate.

Reduction Method for Nitrates.⁴⁷

Reagents.—1. Sodium or potassium hydrate solution. Dissolve 250 grams of the purest hydrate obtainable in 1.25 liters of distilled water and boil down to one liter.

2. Aluminum foil. Use strips about 5 cm. long, .012 mm. thick, and of such a width that each strip weighs about 0.35 gram.

Procedure.—Put 50 c.c. of the sample, or a smaller portion diluted to 50 c.c., in a test tube about 30 cm. long and 15 mm. in diameter. Add five c.c. of the sodium (or potassium) hydrate solution and a strip of the aluminum foil. Place a loose stopper in the mouth of the tube and let stand at room temperature over night. After the reduction is complete put the contents of the tube in a Kjeldahl distilling flask and distill with steam. Cool the distillates and nesslerize in the usual way. If the sample is high in nitrate, dilute with ammonia-free water an aliquot portion of the supernatant liquid in the reduction tube and nesslerize directly.

In this process it is necessary in all cases to correct for free ammonia and nitrites, and where the free ammonia is very high it shall be boiled off before the determination of nitrates is made, restoring to the boiled sample its original volume by adding ammonia-free water.

A control determination, using the same batch of reagents and nitrogen-free water, shall always be made, because the accuracy of the procedures depends largely upon the purity of the reagents used.

TOTAL NITROGEN.

In sewage work it is frequently of assistance to record and study the total nitrogen contents. This is ordinarily done by adding together the nitrogen in the form of organic nitrogen, free ammonia, nitrites and nitrates, each of which is determined as already described.

Sometimes it is desirable or convenient to determine the total nitrogen by a single determination as follows:

Digest the sample in the same manner as directed for the determination of organic nitrogen, stopping the procedure just before the addition of the permanganate. From this point proceed as directed by the Association of Official Agricultural Chemists⁴ for the determination of total nitrogen in fertilizers containing nitrates.

DETERMINATION OF RESIDUE ON EVAPORATION.

TOTAL RESIDUE, OR TOTAL SOLIDS.⁴⁹

Procedure.—Ignite and weigh a clean platinum dish, and into it measure 100 c.c. of the water. If the water is of high magnesium content, add 25 c.c. of $\frac{N}{80}$ sodium carbonate solution to the water, correcting for this addition in the computation. Evaporate to dryness on a water bath. Then heat the dish in an oven (surrounded by boiling glycerin solution or toluene) at a temperature of about 103° C. for one-half hour. Then let it remain in a desiccator over sulphuric acid until cool, and weigh. The increase in weight gives the total solids or residue on evaporation.

This residue in the case of sewages and waters high in organic matter is generally ignited to burn off the organic matter which, with some volatile matters, constitutes the "loss on ignition."

With waters low in organic matter but relatively high in iron, this residue is frequently used as a matter of convenience for the determination of iron.

LOSS ON IGNITION AND FIXED RESIDUE.⁵⁰

Heat the platinum dish containing the residue in a "radiator" which consists of another platinum dish large enough to allow an air space of about half an inch between the inner and outer dishes, the inner dish being supported by a triangle of platinum wire laid on the bottom of the outer dish. Over the inner dish is suspended a disc of platinum foil large enough to cover the outer dish, to radiate the heat into it. The larger dish is heated to bright redness until the residue is white or nearly so. Allow the dish to cool, and moisten the residue with a few drops of distilled water; dry the residue in an oven for half an hour, cool in a desiccator and weigh. This weight gives the fixed solids or fixed residue on evaporation, and the difference between it and the total solids, or the total residue on evaporation, gives the loss on ignition.

The manner in which the residue behaves as to odor and color upon ignition in some cases gives a helpful clue to the character of the organic matter, and in such instances the changes shall be made a matter of record.

SUSPENDED MATTER, OR SUSPENDED SOLIDS.

This determination is made by obtaining the difference between the total solids in the unfiltered portion of a sample and in a portion from which the suspended matters have been removed. For sewages and waters containing suspended matter not too small in size, filtration may be done through a filter paper. For clay-bearing waters, suspended matter is best removed by a Berkefeld filter. Do not use a Pasteur filter. The use of a tarred filter of asbestos in a Gooch crucible^{50a} may be found advantageous, and the suspended matter determined directly.

Treat unfiltered and filtered samples alike as regards the addition or omission of sodium carbonate solution.

The volume⁵¹ of suspended matter in connection with sewage work in England has received considerable attention. In America the subject has not been studied much as yet.

LOSS ON IGNITION AND FIXED RESIDUE DUE TO SUSPENDED AND DISSOLVED MATTERS.

By treating the total residue from the filtered sample in the same manner as above described for the total residue, there is obtained the loss on ignition due to dissolved matters, and by difference the loss on ignition due to suspended matters.

DETERMINATION OF IRON.⁵²

Iron is found in water in dissolved and suspended forms and in both ferrous and ferric conditions, depending upon the nature of the sample. In ground waters the iron is frequently in an unoxidized and soluble condition, partly in combination with organic matter and partly as a carbonate or perhaps sulphate. Silt-bearing waters contain much iron in suspension and in an oxidized form. Sewages and sewage effluents, particularly those associated with manufacturing wastes, contain various forms and combinations of iron of different degrees of solubility and oxidation.

TOTAL IRON.⁵³

Reagents.—1. Standard iron solution. Dissolve 0.7 gram of crystallized ferrous ammonium sulphate in 50 c.c. of distilled water and add 20 c.c. of dilute sulphuric acid. Warm the solution slightly and add potassium permanganate until the iron is

completely oxidized. Dilute the solution to one liter. One c.c. of the standard solution equals 0.0001 gram Fe.

2. Potassium sulphocyanide solution. Dissolve 20 grams of the salt in one liter of distilled water.

3. Dilute hydrochloric acid. One volume of acid (Sp.gr. 1.2) and one volume of distilled water. This shall be free from nitric acid.

4. Potassium permanganate solution $\frac{N}{6}$ 6.30 grams per liter.

Procedure.—Evaporate 100 c.c. of the sample to dryness, or use the residue left after the determination of solids, as previously described. With silt-bearing waters the quantity of iron is sometimes so great that it is necessary to use as little as 10 c.c. of the sample. With such waters evaporation should be made in the presence of 5 to 10 c.c. of strong hydrochloric acid to effect complete solution of the iron. If the sample of water contains much organic matter, destroy this by ignition, taking care not to prolong the ignition so as to render the iron too difficultly soluble.

Cool the dish and add five c.c. of dilute hydrochloric acid to moisten the whole of the inner surface of the dish. Place the dish on the steam bath for two or three minutes and again moisten the whole inner surface of the dish by allowing the hot acid to flow over it. Add 5 to 10 c.c. of distilled water to rinse down the sides of the dish, and again place on the water bath for about three minutes.

The hot acid solution is washed from the dish with distilled water into a 100 c.c. nessler tube. Filter the sample if necessary, carefully washing the filter paper with hot water. Add a drop or two of potassium permanganate solution to oxidize the iron to a ferric condition. The color of the permanganate should persist for at least five minutes; if not, add more permanganate solution, a drop at a time.

To the cooled solution 10 c.c. of potassium sulphocyanide solution are added, and the volume made up to 100 c.c. and well mixed.

Immediately compare the resulting color with that in permanent standards (see below) or in a series of standards prepared side by side with the sample in 100 c.c. nessler tubes in which amounts

of standard iron solution ranging from 0.05 to 4 c.c. are first diluted with water to about 50 c.c. Five c.c. of dilute hydrochloric acid and a drop or two of potassium permanganate are added to each tube of standard solution, and all are diluted to 100 c.c. The number of standards needed is governed by the quantity of iron likely to be present in the sample examined.

Potassium sulphocyanide shall be added to each of the standard solutions at the same time that this reagent is added to the samples of water under examination. Comparison of the sample with the standards, which are made up to 100 c.c. after adding the sulphocyanide and mixing, should be made immediately.

PERMANENT IRON STANDARDS.²⁵

Reagents.—1. Platinum solution. Dissolve 2 grams of potassium platonic chloride in distilled water, add 100 c.c. of strong hydrochloric acid and make up to one liter with distilled water.

2. Cobalt solution. Dissolve 24 grams of dry cobaltous chloride crystals in a small amount of distilled water, add 100 c.c. of strong hydrochloric acid, and make up to one liter with distilled water.

Procedure.—Prepare a series of standards by putting the following amounts of the platinum and cobalt solutions in 100 c.c. nessler tubes and making up to 100 c.c. with distilled water.

TABLE 8.

No. of c.c. Standard Iron Solution	No. of c.c. Platinum Solu- tion	No. of c.c. Cobalt Solution
.0	0	0
.1	2	1.0
.3	6	3.0
.5	10	5.0
.7	14	7.5
1.0	20	11.0
1.5	28	17.0
2.0	35	24.0
2.5	39	32.0
3.0	40	43.0
3.5	40	55.0

Take one of the treated samples, add 10 c.c. of potassium sulphocyanide, mix, and compare *immediately* with the permanent standards. Then proceed in a like manner with the other samples.

TOTAL IRON IN SOLUTION.

Determine, by the same method as above given for total iron, the iron in the sample after filtration. Do not forget that oxidation during filtration may precipitate ferrous iron in some samples. Generally this is not the case.

TOTAL IRON IN SUSPENSION.

This may be determined by taking the difference between the total iron obtained by the above method in the unfiltered sample and the dissolved iron found by the same method in the filtered sample.

FERROUS IRON.⁵⁴

The total ferrous iron shall be determined from an unfiltered sample, and the dissolved ferrous iron from a sample of water which has been freed from matters in suspension.

Reagents.—1. Standard iron solution. Dissolve 0.7 gram of crystallized ferrous ammonium sulphate in one liter of water to which are added 10 c.c. of dilute sulphuric acid. This solution easily oxidizes, and should be freshly prepared when needed. One c.c. of this standard solution contains 0.0001 gram of Fe.

2. Potassium ferricyanide solution. Dissolve five grams of the salt in one liter of distilled water. Use a freshly prepared solution.

3. Dilute sulphuric acid. Dilute one part of sulphuric acid, specific gravity 1.84, with five parts of distilled water.

Procedure.—To 50 c.c. of the sample add 10 c.c. of dilute sulphuric acid and 15 c.c. of potassium ferricyanide solution. The whole is made up to 100 c.c. with distilled water. Before the cyanide solution is added, the suspended matter is removed by filtration if necessary. The color obtained in the sample in the above treatment is compared with standards made from the ferrous iron solution as follows:

Place in 100 c.c. nessler tubes, in the following order, 75 c.c. distilled water, 10 c.c. dilute sulphuric acid, and 15 c.c. potassium ferricyanide solution, and mix well the contents of the tube. Prepare as many tubes in this way as are desired in order to obtain standards comparable to the iron contents of the samples being examined. Add various quantities of standard ferrous

iron solution to several tubes, mix well, and compare the resulting colors with the samples *immediately*.

FERRIC IRON.

The amount of ferric iron both in solution and in suspension is determined by the difference between the total iron and the ferrous iron obtained by the methods already described.

VOLUMETRIC METHOD FOR SAMPLES WITH HIGH IRON CONTENTS.⁵⁵

Some samples of sewages and waters mixed with trade wastes and mine drainage contain so much iron that it is preferable to use the volumetric method given below for the determination of both total and dissolved iron, rather than to work with such small quantities as would permit the application of the colorimetric methods above described.

When iron is present in large quantities in suspension, as in some sewages and septic tank effluents, it may be filtered off and the residue washed, ignited, and fused with potassium and sodium carbonate. The fusion is then extracted with hydrochloric acid.

After the iron is in solution it is reduced with zinc and titrated directly with $\frac{N}{10}$ potassium permanganate solution. The effect of free hydrochloric acid on the free permanganate solution may be avoided by adding manganous sulphate to the solution.

The quantity of iron present may be readily computed from the number of c.c. of $\frac{N}{10}$ potassium permanganate used, each c.c. of which corresponds to 0.0056 gram of iron (Fe).

SEPARATION AND DETERMINATION OF LEAD, ZINC, COPPER, AND TIN.⁵⁶

Lead, zinc, copper, and tin determinations are of importance in connection with the solvent action of some waters upon pipes and other containers. The use of certain "germicides" also involves some of these tests.

The first three may be determined (a) colorimetrically or (b) electrolytically. The former method is not so accurate as a combination of both, and is chiefly of value as a qualitative test.

It is possible to make a rough estimation of the amount of lead present in clear waters by acidifying with acetic acid, saturating with hydrogen sulphide and comparing the color produced with

that produced by standard lead solutions contained in nessler tubes similar to those for containing the sample. This method, however, is not applicable when the water is colored or contains iron, and in those cases the following method is advised:

Reagents.—1. Standard lead solution. To a strong solution of lead acetate add a slight excess of sulphuric acid, filter off and wash the precipitate. Dissolve it in strong ammonium acetate solution. Make up to a known volume and determine the lead in an aliquot part by precipitating with potassium bichromate and weighing the lead chromate. Dilute an aliquot part so that one c.c. of the dilute solution equals 0.001 gram of lead.

2. Ammonium chloride, 25 per cent solution.
3. Ammonia water, specific gravity 0.96.
4. Hydrogen sulphide water.
5. Sulphuric acid, Sp. gr. 1.84.
6. Potassium oxalate crystals.
7. Potassium sulphate crystals.
8. Ammonium acetate, strong solution.
9. Acetic acid, 50 per cent.
10. Alcohol, 95 per cent.
11. Alcohol, 50 per cent.
12. Nitric acid, 1 to 10, and strong acid (Sp. gr. 1.42).
13. Hydrochloric acid, Sp. gr. 1.20.
14. Urea crystals.

PROCEDURE FOR LEAD, ZINC, AND COPPER.

Concentrate (§ 1)* rapidly by boiling in a seven-inch porcelain dish, over a free flame, three or four liters of the sample to be tested—or more if very small amounts of the metals are suspected—to a volume of about 30 c.c. Add 10 or 15 c.c. ammonium chloride reagent to assist in the separation of the sulphides, then add a few drops of strong ammonia and a considerable excess of saturated hydrogen sulphide water. After standing some time, preferably over night, add a little more ammonia and hydrogen sulphide water; boil the contents of the dish a few moments and filter.

* The section numbers in this text description refer to the schematic tables at the end.

The precipitate (§ 2) contains all the lead, zinc, copper and iron as sulphides; also the suspended organic matter. The soluble coloring matter passes into the filtrate (§ 3).

Wash the precipitate a few times with hot water and return the precipitate on the filter to the original dish and boil with dilute nitric acid (1 part acid Sp. gr. 1.42 to 10 parts water), rubbing the sides of the dish with a bit of filter paper, if necessary, to detach any sulphide precipitate which adheres. After filtering, and washing several times with hot water, evaporate the filtrate and washings in the original dish to a bulk of about 10 or 15 c.c., cool, add five c.c. concentrated sulphuric acid, and heat over a lamp until copious fumes of sulphuric acid appear.

At this point if lead and copper are known to be absent and zinc alone is to be determined (§ 13), dilute the contents of the dish slightly; add an excess of ammonia to precipitate iron, and filter. Make the filtrate slightly acid with sulphuric acid; concentrate to a volume of about 150 c.c. and transfer to a weighed platinum dish; add two grams of pure potassium oxalate and 1.5 grams of pure potassium sulphate. Place the dish in circuit on an electrolytic apparatus with a current of about 0.3 ampere, and allow it to remain for three hours.

Siphon off the solution in the dish and at the same time run into the dish a stream of distilled water while the current is still on, in order to expel the free sulphuric acid which might dissolve some of the zinc if the current was broken at first. When the acid is expelled, break the current, remove the dish, wash with water from a wash bottle, then with 95 per cent alcohol free from residue, dry at 70° C., cool and weigh.

The difference between this weight (§ 10) and the weight of the empty platinum dish gives the amount of metallic zinc.

Some difficulty has been experienced in this determination in obtaining reagents which would give a perfect blank. In all cases blank determinations are made with each new lot of reagents and corrections made in the results when necessary.

If lead is present, however, dilute slightly (with water) the contents of the dish, after fuming with sulphuric acid, and treat with 150 c.c. of 50 per cent alcohol, in order to render the lead

sulphate insoluble. After standing some time, preferably over night, filter off the lead sulphate and wash with 50 per cent alcohol.

Dissolve the precipitate by boiling the filter containing the lead sulphate in ammonium acetate solution in a porcelain dish. (§ 4). Filter into a 100 c.c. nessler tube and wash the filter with boiling water containing a little ammonium acetate. Divide this filtrate in halves and treat one-half with saturated hydrogen sulphide water in order to get an approximation of the amount of lead present. To the other half, or an aliquot portion, if a large amount of lead is present, add a few drops of acetic acid, then an excess of saturated hydrogen sulphide water, and compare the color obtained with a set of standards made by treating with hydrogen sulphide water known amounts of standard lead solution.

To each of the standards add also a little acetic acid and ammonium acetate solution.

After thus determining the lead, if zinc is present but no copper, concentrate the filtrate from the lead sulphate to expel the alcohol, and remove the iron by an excess of ammonia. Acidify the filtrate from the iron with sulphuric acid, concentrate and electrolyze for zinc as previously described.

If copper also is present (§ 5), however, concentrate the filtrate from the lead until the alcohol is expelled and add an excess of ammonia.

§ 6. Filter off any iron precipitate.

Neutralize the filtrate (§ 7) with sulphuric acid, add 10 c.c. of concentrated sulphuric acid and one gram of urea, and electrolyze the solution for two hours with a current of about 0.5 ampere. At the end of this period break the circuit, empty the dish and wash the deposit with water, saving the filtrate and washings which contain the zinc.

Dry in a water-oven and weigh; (§ 8) then dissolve the copper off the dish with dilute nitric acid (1 to 10), wash the dish again with water, dry and weigh. The difference in weight gives the copper.

After the copper is all deposited treat the solution containing the zinc with ammonia until nearly all the sulphuric acid is

neutralized, concentrate to slightly less than the capacity of the dish and add potassium sulphate and oxalate, as previously described.

The solution is now ready to be electrolyzed for zinc. This solution, however, is generally saturated with ammonium salts due to neutralizing the large quantity of sulphuric acid, and it is frequently impossible to get the zinc deposited firmly on the dish before the salts begin to crystallize out and interfere. To avoid this difficulty half the solution (diluted so as to fill the dish) may be taken and electrolyzed for zinc, or if the amount of zinc is very low, all of the zinc may be precipitated as sulphide in acetic acid solution, ignited to oxide, and weighed. This difficulty will not occur when copper is absent, as there will not be such an excess of ammonium salts present.

PROCEDURE FOR COPPER ONLY.

When copper alone is to be determined (§ 11), concentrate the original water to small bulk with a little hydrochloric acid and 5 to 10 c.c. of concentrated nitric acid. When near dryness, add 10 c.c. or more of concentrated sulphuric acid, and heat over a lamp until copious fumes of sulphuric acid come off. Dilute with water, boil and filter into a beaker, washing with hot water. Neutralize the filtrate with ammonia, add a slight excess of sulphuric acid and a considerable excess of hydrogen sulphide water; heat to boiling, add more hydrogen sulphide water, and allow to stand some time, preferably over night. Filter off the sulphides. Dissolve the precipitate in dilute nitric acid; (§ 12) fume with sulphuric acid as previously described, and proceed as above described in the case of copper and zinc being present after the removal of the lead.

If zinc is present little or none of it will be precipitated as sulphide, and if any, it will not interfere in the determination of the copper.

If lead is present, practically all of it will be filtered off as sulphate after the first fuming with sulphuric acid, but as a precautionary measure allow the solution which is ready for electrolysis to stand with a slight excess of sulphuric acid present,

before adding the 10 c.c. of acid and the urea. If any precipitate forms, the solution is filtered directly into the platinum dish.

PROCEDURE FOR TIN.

In connection with the above methods it may be remarked that small quantities of tin are occasionally met with in waters that have passed through tin or tin-lined pipes. This metal, if

TABLE 9.

SCHEME FOR THE SEPARATION OF LEAD, ZINC AND COPPER.

<p>§ 1. Concentrate sample. Add 10 c.c. NH_4Cl, a few drops NH_4OH and saturate with H_2S. Allow to stand, add NH_4OH and H_2S. Boil, filter, and wash.</p>			
<p>§ 2. Dissolve the precipitate in dilute HNO_3. Filter and wash. Evaporate to 10 or 15 c.c.; cool; add 5 c.c. concentrated H_2SO_4 and heat until H_2SO_4 is given off. Dilute slightly and treat with 150 c.c. of 50 per cent alcohol. Allow to stand; filter, and wash with 50 per cent alcohol.</p>			<p>§ 3. The filtrate contains the coloring matter. Reject.</p>
<p>§ 4. The precipitate contains the Pb. Dissolve in $(\text{NH}_4)_2\text{AC}$ solution. Filter into a 100 c.c. nessler tube and wash with water containing $(\text{NH}_4)_2\text{AC}$. Divide filtrate in halves. Saturate one half with H_2S. Determine the Pb in the other half by adding AC and H_2S and then comparing with standard containing known amounts of Pb.</p>	<p>§ 5. The filtrate contains the Zn and Cu. Concentrate to expel alcohol. Add excess of ammonia, filter and wash precipitate.</p>		
<p>§ 8. The deposit is Cu. Dry and weigh as Cu.</p>	<p>§ 6. The precipitate contains the Fe. Reject.</p>	<p>§ 7. The filtrate contains the Zn and Cu. Neutralize with H_2SO_4. Add 10 c.c. concentrated H_2SO_4 and 1 g. urea. Electrolyze for two hours with a current of 0.5 ampere. Break circuit. Empty dish and wash.</p>	
	<p>§ 9. The solution contains the Zn. Nearly neutralize with NH_4OH. Concentrate to less than the capacity of the dish. Add 2 g. K_2OX and 1.5 grams of K_2SO_4. Electrolyze for 3 hours with a current of 0.3 ampere. Siphon off solution, break circuit, wash with water, then alcohol, dry at 70°C, cool and weigh.</p>		
<p>§ 10. The weighed residue is metallic Zn.</p>			

TABLE 10.
SCHEME FOR COPPER ONLY.

§ 11. Add HCl and HNO₃ and concentrate. Add 10 c.c. or more concentrated H₂SO₄ and heat until H₂SO₄ volatilizes. Dilute, boil, filter and neutralize with NH₄OH. Add a slight excess of H₂SO₄ and a considerable excess of H₂S water. Heat to boiling, add more H₂S water and allow to stand. Filter off sulphides.

§ 12. The precipitate contains the Cu. Dissolve in dilute HNO ₃ , fume with H ₂ SO ₄ , precipitate iron with NH ₄ OH, and proceed as in Section 7.	Reject the filtrate from the sulphides.
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TABLE 11.
SCHEME FOR ZINC ONLY.

§ 13. Follow scheme for all three metals as given in Table 9 through Section 5. Nearly neutralize the filtrate with H₂SO₄, concentrate to less than the capacity of the dish and electrolyze as directed in Section 9.

present, is removed with the iron by ammonia in the lead, zinc and copper separation; and in the method for copper alone, it is removed in the same way and may be further avoided by dissolving the sulphides in strong nitric acid (Sp. gr. 1.42) when any tin present will be separated as an insoluble compound.

There is as yet no satisfactory method for the quantitative separation of small quantities of tin.

These schematic tables illustrate the procedures given. The figures refer to the section numbers in the text.

DETERMINATION OF HARDNESS.⁵⁷

The hardness of a water is caused by the presence of certain soluble mineral constituents which consist chiefly of the salts of calcium and magnesium. Hardness is commonly measured by the soap-destroying power of the water. The addition of a potassium or sodium soap to a hard water decomposes the soap and produces insoluble soaps of lime and magnesia. The solubility of calcium and magnesium carbonate in a water beyond certain limits depends upon the presence of carbonic acid, and results in what are called bicarbonates. As the carbonic acid is

removed by boiling the water, the normal carbonates of lime and magnesia are in consequence precipitated. The precipitation, however, is not complete, and portions of the calcium and magnesium carbonates still remain dissolved, even after prolonged boiling. To the extent to which these salts are precipitated, the hardness of the water is diminished or softened by boiling. The hardness thus removed is called "temporary hardness." The hardness which still remains after boiling the water is largely due to the sulphates and chlorides of lime and magnesia, and to the carbonates of these bases still held in solution. The hardness not removed by boiling is termed "permanent hardness."

For many years hardness determinations by the soap method have been included in the sanitary analyses of waters for the purpose of rapidly approximating the quantities of calcium and magnesium salts in the water. In more precise terms, these results record the soap-consuming properties of the water. Rapid developments in connection with the coagulation⁸⁸ and purification of muddy water, and developments in the field of water softening,⁸⁹ have combined to direct especial attention to the group of compounds associated with hardness. The more important tests for this allied group are given in this report.

TOTAL HARDNESS BY THE SOAP METHOD.⁹⁰

Reagents.—1, Standard calcium chloride solution.—Dissolve 0.2 gram of pure calcite (calcium carbonate) in a little dilute hydrochloric acid, being careful to avoid loss of solution by spattering. Evaporate it to dryness several times to expel excess of acid. Dissolve in distilled water and make up to one liter. One c.c. is equivalent to 0.0002 gram of calcium carbonate.

2. Standard soap solution.—Dissolve 100 grams of dry white Castile soap in one liter of 80 per cent alcohol, and allow this solution to stand several days before standardizing. From the above stock solution dilute with 70 per cent alcohol such a quantity that the resulting diluted soap solution will give a permanent lather when 6.40 c.c. of it are properly added to 20 c.c. of standard calcium chloride solution. Usually from 75 c.c. to 100 c.c. of the stock soap solution are required for making one liter

of the standard soap solution. Pure potassium soap made from lead plaster and potassium carbonate may be used to advantage in place of Castile soap.

In standardization 20 c.c. of the calcium chloride solution are put into a 250 c.c. glass stoppered bottle and diluted to 50 c.c. with distilled water which has been recently boiled and cooled. There is then added from a burette 0.2 or 0.3 c.c. of soap solution at a time, shaking the bottle vigorously after each addition until a lather over the entire surface of the water is formed which remains continuous for five minutes after the bottle is laid upon its side. When the soap solution is of the strength above stated, then the quantity of calcium carbonate equivalent to each cubic centimeter of the soap solution is indicated in the following table:

TABLE 12
TABLE OF HARDNESS, SHOWING THE PARTS PER MILLION OF CALCIUM CARBONATE (CaCO_3)
FOR EACH TENTH OF A CUBIC CENTIMETER OF SOAP SOLUTION
WHEN 50 C.C. OF THE SAMPLE ARE USED.

c.c. of Soap Solution	0.0 c.c.	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.	0.5 c.c.	0.6 c.c.	0.7 c.c.	0.8 c.c.	0.9 c.c.
0.0								0.0	1.6	3.2
1.0	4.8	6.3	7.9	9.5	11.1	12.7	14.3	15.6	16.9	18.2
2.0	19.5	20.8	22.1	23.4	24.7	26.0	27.3	28.6	29.9	31.2
3.0	32.5	33.8	35.1	36.4	37.7	38.0	40.3	41.6	42.9	44.3
4.0	45.7	47.1	48.6	50.0	51.4	52.9	54.3	55.7	57.1	58.6
5.0	60.0	61.4	62.9	64.3	65.7	67.1	68.6	70.0	71.4	72.9
6.0	74.3	75.7	77.1	78.6	80.0	81.4	82.9	84.3	85.7	87.1
7.0	88.6	90.0	91.4	92.9	94.3	95.7	97.1	98.6	100.0	101.5

This table does not provide for the use of as large a volume of soap solution for a single determination as was formerly the case, owing to the fact that the end point becomes somewhat obscured in the presence of magnesium salts, as explained beyond, when more than about seven c.c. are used.

Procedure.—Measure 50 c.c. of the water into a 250 c.c. bottle and add soap solution in small quantities and in precisely the same manner as described under the standardization of the soap solution. From the result obtained there may be noted from the table above the total hardness of the water in terms of calcium carbonate.

When adding the soap solution to waters containing mag-

nesium salts, it is necessary to avoid mistaking the false or magnesium end-point for the true one.⁶¹ Consequently, after the titration is apparently finished, read the burette and add about 0.5 c.c. of soap solution. If the end point was due to magnesium, the lather now disappears. Soap solution must then be added until the true end point is reached. Usually the false lather persists for less than five minutes.

When more than seven c.c. of soap solution are required for 50 c.c. of the water, it is necessary to take less of the sample and dilute to 50 c.c. with distilled water which has been recently boiled and cooled. This step reduces somewhat the disturbing influence of magnesium salts,⁶² which consume more soap than do equivalent weights of calcium salts.

At best the soap method is not a precise test on account of the varying amounts of calcium and magnesium present in different waters. For hard waters, especially in connection with processes for purification and softening, it is advised that this method be not used.

When the same water is frequently analyzed, it may be of assistance to standardize the soap solution against a mixture of calcium and magnesium salts, the relative proportions of which approximate those found in the water.

When free carbonic acid is present in the sample in considerable amount, it should be removed by aëration.

The strength of the soap solution should be determined from time to time, to make sure that it has not materially changed while standing. Unless otherwise stated, record all results in terms of calcium carbonate.

English degrees of hardness, Clark's scale, are equivalent to grains of calcium carbonate per imperial gallon, and are multiplied by 14.3 to give parts per million.

French degrees of hardness represent parts per 100,000 of calcium carbonate, and are multiplied by 10 to give parts per million.

German degrees of hardness represent parts per 100,000 of calcium oxide, and are multiplied by 17.8 to give parts of calcium carbonate per million.

TOTAL HARDNESS BY INCRUSTANT METHOD.⁶²

This method gives fairly satisfactory results for hard waters. It consists in adding standard sulphuric acid to the sample until the alkalinity is neutralized. See Alkalinity, p. 61. Then the incrustants (in which form all of the calcium and magnesium are present) are determined with the aid of soda reagent by the procedure detailed on p. 65.

TOTAL HARDNESS BY COMPUTATION FROM CALCIUM AND MAGNESIUM CONTENTS.

The most accurate method of ascertaining total hardness is by computation from the results of determinations of calcium and magnesium in the water, which may be obtained by the methods noted below. The calcium should be computed as calcium carbonate, and the magnesium should also be calculated ordinarily to an equivalent quantity of calcium carbonate, although in connection with some water softening problems the magnesium is preferably calculated to magnesium carbonate.

For purposes of ordinary analysis it is not considered by the committee that it is necessary to obtain the total hardness by this means, although, if waters behave somewhat abnormally with soap solutions, as do magnesium waters, it is desirable as a control, especially in the case of waters with which the analyst is not familiar.

In connection with water softening projects, the determination of the total hardness by this method at intervals is regarded as imperative, as a control for the method above.

CALCIUM BY THE GRAVIMETRIC METHOD.⁶⁴

After the removal of silica, iron and alumina from a known volume of the water, add to the filtrate an excess of ammonium oxalate, filter off the calcium oxalate, ignite and weigh as calcium oxide in accordance with regular procedures for calcium determinations, and compute the result in parts of Ca per million.

CALCIUM BY THE VOLUMETRIC METHOD.⁶⁵

Calcium may be determined volumetrically with equally satisfactory results and somewhat more quickly by washing the precipitate of calcium oxalate obtained as before with as little hot water as possible, and then dissolving it in dilute sulphuric acid and titrating with a standard solution of potassium permanganate.

MAGNESIUM BY THE GRAVIMETRIC METHOD.⁶⁴

Concentrate the filtrate from which the calcium has been removed; add sodium phosphate and ammonia, and allow the solution to stand for 12 hours in a cool place; then filter, ignite the precipitate and weigh as magnesium pyrophosphate, and compute the results in parts of Mg per million.

Magnesium in the filtrate from the calcium oxalate precipitate may also be determined by turbidimetric⁶⁶ comparison with standards of magnesium sulphate.

MAGNESIUM BY THE VOLUMETRIC METHOD.⁶⁷

Measure 100 c.c. of the water into a six-inch porcelain dish. Add exactly enough $\frac{N}{80}$ sulphuric acid solution to make the water neutral to lacmoid or erythrosine, as found by the determination of the alkalinity (see page 61). Boil down to a volume of about 30 or 40 c.c. to expel the free, half-bound and bound carbonic acid and to concentrate the solution.

Introduce by means of a pipette 25 c.c. of a clear saturated solution of lime water of known strength into a 150 c.c. glass stoppered flask. While still hot, transfer immediately to this flask the water concentrated as above described. Rinse out the porcelain dish with hot boiled distilled water and make up the solution to about two c.c. above the 150 c.c. line in the flask. Mix well, stopper immediately and cool.

When the precipitated magnesium hydrate has settled completely, pipette off very carefully, in order not to disturb the precipitate at the bottom of the flask, 50 c.c. of the clear solution. Run this solution into a known quantity of $\frac{N}{80}$ sulphuric acid, which is not quite sufficient to neutralize it. Finish the titration by adding more acid, using phenolphthalein as an indicator.

If C represents the number of c.c. of $\frac{N}{80}$ sulphuric acid required to neutralize 25 c.c. of the lime water, and N the number of c.c. of the same acid required to neutralize the excess of lime water in the sample, then Mg in parts per million equals $2.4 (C-3N)$.

It is to be borne in mind that the strength of lime water solutions varies somewhat with the temperature. Where especially accurate data are required a control determination should be

made by treating the specified quantity of lime water with distilled water in the same manner as in the regular procedure.

MAGNESIUM BY THE SOAP METHOD AFTER REMOVAL OF CALCIUM.⁶⁸

Approximate determinations of magnesium salts present may be obtained by shaking up about 0.1 gram of solid ammonium oxalate with 100 c.c. of the water to be examined. Then put the whole on a water bath; cool and filter through a dry filter paper; discard the first 25 c.c. of the filtrate and then titrate 50 c.c. of the remaining filtrate with soap solution in the usual way.

The amount of magnesium (Mg) is computed as 24 per cent of the hardness indicated in terms of calcium carbonate by Table 12.

The results may be made of somewhat greater accuracy if the soap solution is standardized against a solution of magnesium sulphate of known strength. In view of the different quantities of soap consumed by equivalent amounts of calcium and magnesium, this step is recommended when a special study is being made of a particular water.

TEMPORARY HARDNESS BY THE SOAP METHOD.

Temporary hardness by the soap method is sometimes estimated by the difference between the total hardness obtained as above described and the permanent hardness obtained by applying the soap test to the water after boiling. This method is not advised.

A known volume of the water is boiled gently for half an hour and the water is then allowed to cool. Recently boiled and cooled distilled water is then added so as to restore the water to its original volume. Volumetric flasks may be used for this purpose. The water is then filtered and the hardness of the filtrate determined by the soap method in the usual manner.

TEMPORARY HARDNESS BY TITRATION WITH ACID.

This is by far the most accurate method of determining the true temporary hardness, or the bicarbonate alkalinity, of all ordinary waters. Alkalinity by the method described below is determined in the original sample of water and also in the water after boiling and filtering in the manner stated in the last paragraph. The difference between the two, if any, is the correct temporary hardness.

For those ground waters containing much iron in the form of bicarbonates, the iron in this form is included as a part of the temporary hardness.

ALKALINITY.⁹⁹

The alkalinity of natural waters ordinarily includes the carbonates and bicarbonates of calcium and magnesium. In some waters in the West it also includes the carbonates of sodium or potassium. Waters that are being softened contain at certain stages alkalinity due to calcium hydrate (lime water), and also sodium carbonate (soda ash).

Procedure with Lacmoid.—Measure 100 c.c. of the water into a porcelain evaporating dish and add 0.5 c.c. of lacmoid solution (two grams in one liter of 50 per cent alcohol). Add $\frac{N}{50}$ sulphuric acid to the water from a burette until within one or two c.c. of the amounts necessary for neutralization have been added. The dish is then placed on a tripod and the contents heated until bubbles of steam begin to break at the surface of the water. The dish is then removed and the titration continued until a drop of the acid striking the surface of the liquid and sinking to the bottom of the dish produces no change in the uniform reddish or purple color of the solution.

The number of c.c. of $\frac{N}{50}$ sulphuric acid used when multiplied by ten gives the number of parts per million of alkalinity in terms of calcium carbonate.

Procedure with Erythrosine.—Where it is desired not to use heat or where heat is not available, satisfactory results may be obtained by measuring 100 c.c. of the water to be examined into a 250 c.c. white glass stoppered bottle with 2.5 c.c. of erythrosine solution (0.1 gram of the sodium salt in one liter of distilled water) and 5 c.c. of chloroform (neutral to erythrosine). Sulphuric acid ($\frac{N}{50}$) is added in small quantities, shaking the bottle vigorously after each addition of the acid. The rose color gradually disappears and is finally entirely discharged by a drop or two of the acid. A white paper held back of the bottle facilitates the detection of any trace of color remaining as the end point is approached. The calculation is the same as with lacmoid.

Notes.—Phenacetolin and rosolic acid behave in a manner similar to lacmoid.

Methyl orange is also used by some analysts in determining alkalinity. With this indicator it is unnecessary to heat the water to drive off free carbonic acid, such as is the case with lacmoid, phenacetolin and rosolic acid. In this regard methyl orange behaves like erythrosine. Many unsatisfactory experiences have been encountered with the use of methyl orange owing to the difficulty with which satisfactory brands of the product may be obtained, and further owing to complications when it is used with waters which are highly turbid or highly colored. Another disadvantage of its use in laboratories connected with water purification plants where coagulants are used is that it is not a "reversible" indicator, that is, it does not react acid to sulphate of alumina or sulphate of iron, as is true of lacmoid. The use of methyl orange is not recommended by the committee, although it is recognized that it is possible with many waters to get accurate alkalinity results from its use in skilled hands.

Alkalinity due to Normal Carbonates.⁷⁰

Normal carbonates of the alkaline earth metals (or the alkalies) are present in a water when the phenolphthalein alkalinity (determined in the cold) is equal to or less than one-half of the alkalinity by lacmoid or erythrosine.

When phenolphthalein is used as an indicator in the cold, one-half of the normal carbonate alkalinity is indicated upon titration with standard sulphuric acid. Only one-half of the quantity present is indicated, because sulphuric acid causes the liberation of an equivalent amount of carbonic acid, which reacts acid to this indicator.

When phenolphthalein gives no color in the cold with a water which is alkaline to lacmoid or erythrosine, it shows that no normal carbonates are present, and that the alkalinity is wholly due to bicarbonates. See Carbonic Acid, page 73.

When the phenolphthalein alkalinity is equal to just one-half the alkalinity by lacmoid or erythrosine, it shows that all of the alkalinity is present in the form of normal carbonates.

When a water has been treated with an excess of lime water only small quantities of normal carbonates remain. The latter are measured by subtracting the phenolphthalein alkalinity from the alkalinity by lacmoid or erythrosine and multiplying the remainder by two.

Alkalinity due to Sodium or Potassium Carbonates.

Waters from alkaline regions which contain sodium or potassium carbonate are found to contain all of their calcium and magnesium as carbonates or bicarbonates. That is, they possess either no incrustants (sulphates, nitrates or chlorides of calcium and magnesium) or only traces of them.

The most accurate method is to determine the total alkalinity by titration with $\frac{N}{50}$ sulphuric acid, using erythrosine or lacmoid as an indicator; then determine the calcium and magnesium contents; and subtract from the total alkalinity the computed alkalinity due to the calcium and magnesium expressed in terms of calcium carbonate. The remainder is the alkalinity due to carbonates of sodium and potassium.

This determination may also be made by applying the method, described beyond, for "incrustants with soda reagent" and noting the excess of acid required to neutralize the alkaline carbonates originally present.

With present information as to solubilities of the normal carbonates of calcium and magnesium, it is difficult in their presence to measure slight quantities of carbonates of sodium or potassium.

Alkalinity due to Hydrates (Caustic Alkalinity).¹⁰

When waters contain calcium or other alkaline hydrate as well as normal alkaline carbonates, the phenolphthalein alkalinity is more than one-half of that with lacmoid or erythrosine.

Determine the alkalinity with phenolphthalein in the cold, and the total alkalinity with erythrosine as an indicator. Do not use lacmoid as the heat required modifies the reactions.

The alkalinity by the former indicator subtracted from that by the latter and multiplied by two gives the normal carbonate alkalinity; this subtracted from the total alkalinity gives the hydrate (or caustic) alkalinity.

When the phenolphthalein alkalinity is less than one-half of that by lacmoid or erythrosine, then no caustic alkalinity is present—the alkalinity being due to carbonates and bicarbonates. See Carbonic Acid, page 73.

When the phenolphthalein alkalinity is the same as that by lacmoid or erythrosine, then all of the alkalinity is due to caustic alkali.

*Silver Nitrate Test for Caustic Alkalinity.*⁷¹

In the operation of water softening plants use is sometimes made of a dilute silver nitrate solution in testing the water for the presence of caustic alkalinity (lime water). A positive result is shown by the appearance of a grayish-brown color.

The evidence before the committee indicates that a positive result may not be obtained until the alkalinity in the form of a hydrate is present to the extent of about 10 parts per million. For this reason, and on account of the color complications arising when chlorides are present in considerable quantities, the use of this test is not advised where accurate data are desired.

PERMANENT HARDNESS BY THE SOAP METHOD.

Reference has already been made to this determination in connection with the temporary hardness above described. The soap-consuming properties of a water which has been thoroughly boiled and filtered, while indicating what is properly called "permanent hardness," does not have much practical significance because it includes in addition to the sulphates and chlorides of calcium and magnesium, also the carbonates of these bases, which are naturally soluble in a water free from carbonic acid. The practice of this method and the use of the expression "permanent hardness" obtained by the soap method are so unsatisfactory that it is advised they be discontinued.

In this connection it is to be pointed out that incrustants do not occur, except perhaps as slight traces, in natural waters containing carbonates of sodium and potassium, although such waters would show a permanent hardness due to normal carbonates of calcium and magnesium.

INCRUSTANTS BY THE SOAP METHOD.

By incrustants is meant, generally speaking, the sulphates, chlorides and nitrates of calcium and magnesium. They may be determined, for waters which are soft or moderately hard, in apparently a fairly satisfactory manner by deducting the total alkalinity from the total hardness by the soap method.

With waters which are quite hard, particularly such as contain considerable quantities of magnesium, this method is not advised.

INCRUSTANTS BY TITRATION WITH EXCESS OF SODIUM CARBONATE.⁶³

This method consists in evaporating to dryness in a platinum dish 100 c.c. of the water together with 50 c.c. of an $\frac{N}{25}$ solution of sodium carbonate. The residue is heated over a flame at a temperature of red heat for five minutes, when the dish is cooled and the residue treated with boiled distilled water to make up the volume to 100 c.c. and allowed to settle; pipette off 25 c.c. of the clear supernatant liquid and determine the excess of sodium carbonate with $\frac{N}{50}$ sulphuric acid, using erythrosine as an indicator.

This method has been given a thorough trial at numerous places in this country, and the concensus of opinion is that the results are quite uniformly too low on account of the solubility of the carbonates of calcium and magnesium. By the use of suitable correction factors, approximate results can probably be obtained with hard waters, but the use of the method according to the evidence now available is not advised.

INCRUSTANTS BY TITRATION WITH EXCESS OF "SODA REAGENT."⁶⁴

The substitution for sodium carbonate in the method last described of the "soda reagent" appears to be a step in the right direction in determining incrusting constituents in hard waters, as it makes less soluble the salts of calcium and magnesium. It does not correct entirely, however, the error due to solubility, and it is advised that, in connection with important work in water softening, gravimetric determinations be made to ascertain the quantities of calcium and magnesium which go into solution and for which a correction factor should be applied under the regular conditions of practice, as follows:

Procedure.—Measure 200 c.c. of the water into a Jena glass Erlenmeyer flask; boil 10 minutes to expel free carbonic acid, and add 25 c.c. $\frac{N}{10}$ "soda reagent" (equal parts of NaOH and Na_2CO_3). Boil to a volume of 100 c.c. cool, rinse into a 200 c.c. graduated flask and make up to 200 c.c. with boiled distilled water. Filter, rejecting the first 50 c.c. and titrate 100 c.c. of the filtrate for excess of soda reagent, using $\frac{N}{20}$ sulphuric acid and erythrosine as an indicator.

If $S = \text{c.c. } \frac{N}{20} \text{ H}_2\text{SO}_4$ equivalent to soda reagent used, and $N = \text{c.c. } \frac{N}{20} \text{ H}_2\text{SO}_4$ required for the excess (back titration), then the

incrustants in parts per million of calcium carbonate equal 12.5 (S-2N).

Water containing carbonates of sodium and potassium will require a larger amount of acid to neutralize the sample after it has been treated than is required to neutralize the volume of soda reagent originally added.

Where the total hardness is determined by this method add to 200 c.c. of the sample a sufficient quantity of $\frac{N}{50}$ sulphuric acid to neutralize the alkalinity in accordance with the result of the latter determination (see page 61). The calcium and magnesium present in the sample as carbonates and bicarbonates are thus converted into sulphates, and the incrustants correspond to the total hardness. Boil down to 100 c.c., add 25 c.c. $\frac{N}{10}$ soda reagent and again boil down to 100 c.c. Cool, rinse into a 200 c.c. flask and make up to 200 c.c. with freshly boiled distilled water. Titrate an aliquot portion and compute as above described the total hardness. The incrustants are of course obtained by subtracting the alkalinity from the total hardness.

Unless a water is very hard, it seems best, in using the "soda reagent" method, to obtain the incrustants by difference, as explained in the last paragraph rather than directly. The percentage error due to solubility is thus reduced.

DETERMINATION OF CHLORINE.¹²

Chlorine in waters and sewages has its origin for the most part in the common salt, which comes, generally speaking, from mineral deposits in the earth, from the ocean vapors carried inland by the wind, or from polluting materials like sewage and trade wastes, both of which contain the salt used in the household and in manufacturing. Comparison of the chlorine contents of a water with that of other waters in the general vicinity known to be unpolluted frequently affords useful information as to its sanitary quality.

Reagents.—1. Standard salt solution. Dissolve 16.48 grams of fused sodium chloride in one liter of distilled water. Dilute 100 c.c. of this stock solution to one liter in order to obtain a standard solution each c.c. of which contains .001 gram of chlorine.

2. Silver nitrate standard. Dissolve about 2.40 grams of silver nitrate crystals in one liter of distilled water. One c.c. of this will contain approximately .0005 gram of chlorine. Standardize this against the standard salt solution.

3. Potassium chromate. Dissolve 50 grams of neutral potassium chromate in a little distilled water. Add enough silver nitrate to produce a slight red precipitate. Filter and make up the filtrate to one liter with distilled water.

4. Aluminum hydrate. Electrolyze ammonia-free water, using aluminum electrodes. Wash the precipitate formed until free from chlorine, ammonia and nitrites. Or, dissolve 125 grams of potash or ammonia alum in one liter of distilled water. Precipitate the aluminum hydrate by cautiously adding ammonium hydrate. Wash the precipitate in a large jar by the successive addition of distilled water and by decantation until free from chlorine, nitrites and ammonia.

Procedure.—Use 50 c.c. of the sample in a white six-inch porcelain evaporating dish for this determination where the chlorine content is not extremely low or very high. If the chlorine is very high in amount, use 25 c.c. or even a smaller quantity if desired, diluting the volume taken with distilled water to 50 c.c. When the sample is very low in its chlorine content, more accurate results may be obtained by using 50 c.c. of the sample and adding, prior to titration, one c.c. of standard salt solution.

Some analysts prefer in testing samples low in chlorine content to concentrate the sample by evaporation. Where evaporation is resorted to care should be taken to avoid the loss of hydrochloric acid by the decomposition of magnesium chloride. This can be accomplished by the addition of a little sodium carbonate, 0.002 gram ordinarily being sufficient.

Chlorine is determined by some observers by extracting with hot distilled water the residue contained in the platinum dish in the determination of the residue on evaporation and proceeding as above described. This is permissible provided care is taken to add sodium carbonate as above indicated to guard against the loss of hydrochloric acid through the decomposition of magnesium chloride.

If the sample has a color greater than about 30 it shall be decolorized by heating it to the boiling point with washed aluminum hydrate (3 c.c. to 500 c.c. of the sample). Make the determination on the portion of the clarified sample, filtered if necessary.

If the sample is acid, neutralize with sodium carbonate; if alkaline due to hydrates, add dilute sulphuric acid until the cold liquid will just discharge the color of phenolphthalein.

Rotate the liquid in the dish to make sure that no portion of the residue on the side walls of the dish remains undissolved; if necessary, use a rubber tipped glass rod to assist in this process.

Add one c.c. of the potassium chromate solution as an indicator. Titrate with the silver nitrate solution, under similar conditions, as to volume, light and temperature as were used in standardizing the silver nitrate. The detection of the end-point is facilitated by frequent comparison of the contents of the porcelain dish in which the determination is being made with those of another dish placed alongside and containing the same quantity of chromate solution in 50 c.c. of distilled water. Where especially accurate work is desired, some analysts prefer to make these observations in a dark room provided with a yellow light.

More accurate results can be obtained for the error due to variations in the volume of the liquid and precipitate, according to the following formula:⁷³ $X = .003V + .02$, where X = the correction in c.c. of silver nitrate solution and V = c.c. of liquid at the end of the titration. Also, if necessary, make corrections⁷⁴ or modifications in treatment for the presence of sulphides and sulphocyanates.

Titration may be made by using 50 c.c. or 100 c.c. samples in nessler tubes,⁷⁵ provided the solutions are standardized under the same conditions.

DETERMINATION OF SULPHURIC ACID.

In some special problems it is desirable to know the quantity of sulphuric acid present in the water. Generally speaking, however, experience during the past decade in this country with this determination has indicated that the value of the results obtained

is not ordinarily commensurate with the labor involved. The reason of this is that it gives only the total quantity present and does not separate the sulphuric acid present as free acid or as the sulphates of the alkaline earth bases or the sulphates of sodium and potassium, or the sulphates of aluminum and iron. These determinations from a practical point of view are best attacked along the lines of acidimetry, incrustants and special tests for sulphates of iron and alumina as described elsewhere in this report.

GRAVIMETRIC METHOD.⁷⁶

Where very accurate results are desired this method is to be preferred, especially for waters low in sulphates. Measure a certain volume of water into a porcelain evaporating dish, the quantity of water depending somewhat upon the probable quantity of sulphuric acid present. Add a slight excess of hydrochloric acid and evaporate to a volume of 250 c.c. if more than this is used. If silica or iron is present, or if the concentrate is not clear, add ammonia to a slight excess, boil and filter. If the sample of water is previously filtered through a Berkefeld filter tube, the precipitation with ammonia may often be omitted. Sulphuric acid is determined by precipitation with barium chloride in the usual way and the sulphuric acid computed from the weight of barium sulphate and expressed in parts of SO_4 per million.

VOLUMETRIC METHOD.⁷⁷

This method is most applicable to waters fairly high in sulphates. It consists in adding an excess of barium chloride to the water, precipitating the excess of barium with a known excess of neutral potassium ammonium chromate, determining the excess of the latter colorimetrically by comparing the color in the sample with the standards containing known quantities of chromate solution.

Reagents.—Solutions ($\frac{N}{10}$) of barium chloride and of potassium ammonium chromate. To prepare the latter dissolve 7.37 grams of pure potassium bichromate in distilled water, neutralize with ammonia and dilute to one liter. Prepare these solutions so that 100 c.c. of each when boiled together produce a yellow precipitate with a supernatant liquid which is colorless and free from barium.

Otherwise, adjust the chromate solution to conform to the strength of the barium chloride solution.

Procedure.—Boil 100 c.c. of the sample for half an hour if necessary to free it from an excess of alkaline earth carbonates, meanwhile maintaining the liquid at a constant volume with frequent additions of distilled water to prevent the precipitation of sulphates. The sample should be neutral; if not, neutralize by the addition of hydrochloric acid. Filter into a 150 c.c. volumetric flask and add 10 to 20 c.c. of barium chloride according to the amount of sulphuric acid present, and boil for a few minutes.

Add a known volume of chromate solution so that the supernatant liquid is slightly yellow with an excess of chromate. Cool, make up to 150 c.c. and filter off 100 c.c. of the contents of the flask into a nessler tube. Estimate the excess of chromate in the filtrate by comparison with a set of standards made by taking different known volumes of chromate solution and diluting to 100 c.c. portions with distilled water in nessler tubes.

Multiply the reading obtained by 1.5 and subtract the result from the volume of chromate used; and subtract the remainder from the amount of barium chloride added. This remainder equals the number of c.c. of standard barium chloride solution used, and when multiplied by 48 gives the parts per million of sulphuric acid as SO_4 .

DETERMINATION OF ACIDITY DUE TO MINERAL ACIDS OR TO THEIR IRON OR ALUMINUM SALTS.⁷⁸

In mining regions waters frequently contain high quantities not only of carbonic acid but also of sulphuric acid and various sulphates—those of iron and aluminum giving an acid reaction.

The total acidity due to free carbonic acid, free sulphuric acid and to sulphates of iron and aluminum is determined by titrating the water in the cold with standard sodium carbonate, using phenolphthalein as an indicator.

The acidity due to free sulphuric acid is determined by titrating the water in the cold with standard sodium carbonate, using methyl orange as an indicator. Some analysts prefer to treat the water with a known excess of standard sulphuric acid, boil for 20

minutes in a porcelain dish, cool and titrate as just described to note the increase in acidity.

The acidity due both to free sulphuric acid and to the sulphates of iron and alumina is determined by titrating the water in the cold with standard sodium carbonate, using erythrosine as an indicator.

By determining the amount of iron present in the sample in the ferrous and ferric condition, according to the last of the methods already described under "Iron" the acidity due to sulphate of alumina and to sulphate of iron is estimated by difference. By determining the amount of ferrous and ferric iron as already described (see pages 47 and 48) the ferrous sulphate and the ferric sulphate may be computed.

From the data obtained as above described and from the methods of computation already given, there may be computed the quantities of each kind of acidity, expressed in parts per million of free carbonic acid and of sulphate acidity in terms of SO_4 ,—both for the free sulphuric acid and the sulphates of iron and aluminum.

DETERMINATION OF ALUM OR SULPHATE OF ALUMINA.

Where these chemicals are used for the coagulation of muddy or highly colored waters, it is essential that care be taken in operating the plant to prevent any of these undecomposed salts appearing in the water after treatment. There are two ways by which a test for their presence is made, one being the measure of acidity of the water when titrated with such indicators as lacmoid or erythrosine, and the other the so-called "logwood" test.

ACIDITY BY LACMOID OR ERYTHROSINE.⁷⁹

This determination is made in a corresponding manner to the alkalinity determination already described on page 6. If the reaction is not alkaline, then $\frac{N}{80}$ sodium carbonate may be used in measuring the amount of acidity due to the alum or to the sulphate of alumina, and the results expressed in terms of calcium carbonate (or computed into grains of sulphate of alumina per gallon).

Lacmoid, phenacetolin or erythrosine may be safely used as

indicators for this test. Methyl orange cannot be used, because as already explained it is not reversible, and indicates only on the alkaline side of the neutral point.

LOGWOOD TEST.⁸⁰

This test is based upon the fact that solutions of logwood or of hematoxylin in the presence of alum and in an acid solution give a well defined reddish or brownish color, the intensity of which can be estimated by comparing the color produced by the sample with a series of standard colors prepared by treating in a corresponding manner a series of solutions containing known quantities of alum or sulphate of alumina.

This test has been widely experimented with, and while it has given satisfactory results in some hands, the consensus of opinion is that it is a difficult test to apply satisfactorily under many conditions in practice. This is due to the fact that it has to be carried out in an acid solution and the acid so supplied has a solvent action upon minute particles of colloidal aluminum hydrate (and perhaps on aluminum silicate) such as are present in very minute quantities in numerous samples of filtered or subsided water. An imperfectly clarified water may thus give erroneously a positive test for sulphate of alumina or alum.

As a means of testing samples for the presence of suspended aluminum hydrate this method is of value when the water is alkaline to lacmoid or erythrosine.

DETERMINATION OF CARBONIC ACID.⁸¹

Carbonic acid may exist in water in three forms, namely, as free, half-bound and bound carbonic acid. Free carbonic acid requires a separate determination, but the remaining forms in which carbonic acid is present may ordinarily be computed from the alkalinity determinations already described.

FREE CARBONIC ACID.

Reagents.—Standard $\frac{N}{10}$ solution of sodium carbonate. Dissolve 2.41 grams of dry sodium carbonate in one liter of distilled water which has been boiled and cooled in an atmosphere free from carbonic acid. Preserve this solution in bottles of resistant

glass, protected from the air by tubes filled with soda-lime. One c.c. equals 0.001 gram of CO_2 .

Procedure.—Measure 100 c.c. of the sample into a tall narrow vessel, preferably a 100 c.c. nessler tube, and titrate rapidly with the $\frac{N}{10}$ sodium carbonate solution, stirring gently until a faint but permanent pink color is produced by phenolphthalein.

The number of c.c. of $\frac{N}{10}$ sodium carbonate solution used in titrating 100 c.c. of water, multiplied by 10, gives the parts per million of free carbonic acid as CO_2 .

Owing to the ease with which free carbonic acid escapes from water, particularly when present in considerable quantities, it is highly desirable that a special sample²² should be collected for this determination, which should preferably be made on the ground. If the analysis cannot be made on the ground, approximate results from water not high in free carbonic acid may be obtained from samples collected in bottles which are completely filled so as to leave no air space under the stopper.

HALF-BOUND CARBONIC ACID.

When a water is acid to phenolphthalein, the half-bound acid is equal to 44 per cent of the alkalinity when the latter is expressed in terms of calcium carbonate.

When a water is alkaline to phenolphthalein, titrate 100 c.c. of the sample with $\frac{N}{10}$ sulphuric acid, using phenolphthalein as an indicator.

Half-bound carbonic acid, indicating bicarbonates, is present only when the alkalinity by phenolphthalein is less than one-half of that by lacmoid or erythrosine. (See page 62).

Then twice the number of c.c. of $\frac{N}{10}$ sulphuric acid required when phenolphthalein is used, subtracted from the number of c.c. of $\frac{N}{10}$ acid used in determining the alkalinity of 100 c.c. of the water with lacmoid or erythrosine as an indicator (see page 61), multiplied by 4.4, gives in parts per million the half-bound carbonic acid as CO_2 .

BOUND CARBONIC ACID.

Compute this in parts per million of CO_2 as 44 per cent of the alkalinity by lacmoid or erythrosine when the latter is expressed in terms of calcium carbonate. (See page 61).

DETERMINATION OF DISSOLVED OXYGEN.

There are three methods in use for the determination of atmospheric oxygen dissolved in water, viz., those of Winkler,⁸² Thresh⁸⁴ and Levy.⁸⁵ Each of these methods has its own particular field of usefulness. All are capable of giving sufficiently accurate results.

The Winkler method is in the most common use in this country, and possesses the advantage of requiring only simple and not readily breakable apparatus. It is therefore recommended as the standard method.

The method of Thresh is perhaps slightly more accurate than the Winkler method, but the apparatus is not so well adapted to field work. For certain purposes, however, as, for example, the determination of dissolved oxygen before and after incubation, it is more practical than the Winkler method, because the apparatus allows the taking of representative samples direct from bottles or other containers.

What is true of the Thresh method is also true to a great degree of the Levy method. With both of these methods the samples are taken in a special, stoppered, separatory funnel.

Reagents.—1. Manganous sulphate solution: Dissolve 48 grams of manganous sulphate in 100 c.c. of distilled water.

2. Solution of sodium hydrate and potassium iodide: Dissolve 360 grams of sodium hydrate and 100 grams of potassium iodide in one liter of distilled water.

3. Sulphuric acid. Specify gravity 1.4 (dilution 1:1).

4. Sodium thiosulphate solution. Dissolve 6.2 grams of chemically pure recrystallized sodium thiosulphate in one liter of distilled water. This gives an $\frac{N}{40}$ solution each c.c. of which is equivalent to .0002 gram of oxygen or 0.1395 c.c. of oxygen at 0° C. and 760 mm. pressure. Inasmuch as this solution is not permanent it should be standardized occasionally against an $\frac{N}{40}$ solution of potassium bichromate as described in almost any work on volumetric analysis. The keeping qualities of the thiosulphate solution are improved by adding to each liter 5 c.c. of chloroform and 1.5 grams of ammonium carbonate before making up to the prescribed volume.

5. Starch solution. Mix a small amount of clean starch with cold water until it becomes a thin paste, stir this into 150 to 200 times its weight of boiling water. Boil for a few minutes, then sterilize. It may be preserved by adding a few drops of chloroform.

Collection of the Sample.—The sample shall be collected with extreme care in order to avoid the entrainment of any oxygen from the atmosphere. The sample bottle shall be preferably a glass stoppered bottle which has a narrow neck and which holds at least 250 c.c. The exact capacity of the bottle shall be determined and for convenient reference this may be scratched upon the glass with a diamond.

If the sample is to be collected from a tap the water shall be made to enter the bottle through a glass or rubber tube which reaches to the bottom of the bottle, the water being allowed to overflow for several minutes, after which the glass stopper is carefully replaced so that no bubble of air is caught beneath it.

If the sample is to be collected from the surface of a pond or tank two bottles shall be used, the ordinary sample bottle and a second bottle of four times the capacity. Both bottles shall be provided with temporary stoppers of double perforation and in both cases a glass tube shall extend through one hole of the stopper to the bottom of the bottle and a short glass tube shall enter the other hole of the stopper but not project into the bottle. The short tube of the sample bottle shall be connected with the long tube of the larger bottle. In collecting the sample the sample bottle shall be immersed in the water and suction applied to the short tube of the large bottle and enough water drawn through the hole to fill the large bottle. In this way the water in the smaller bottle will be changed several times and a fair sample secured.

If the sample is to be taken at a depth below the surface both bottles may be connected, lowered to the desired depth, and if the smaller bottle is placed beneath the larger one the water will enter the small bottle and pass from that into the larger bottle, the air escaping from the short tube of the large bottle. As soon as the small bottle has been filled remove the temporary stopper

and insert the permanent glass stopper using care not to entrain any bubbles of air.

Procedure.—Remove the stopper from the bottle and add approximately two c.c. of the manganous sulphate solution and two c.c. of the sodium hydrate-potassium iodide solution delivering both of these solutions beneath the surface of the liquid by means of a pipette. Replace the stopper and mix the contents of the bottle by shaking. Allow the precipitate to settle. Remove the stopper, add about two c.c. of sulphuric acid and mix thoroughly. Up to this point the procedure shall be carried on in the field but after the sulphuric acid has been added and the stopper replaced there is no further change and the rest of the operation may be conducted at leisure. For accurate work there are a number of corrections necessary to be made, but in actual practice it is seldom necessary to take them into account as they are ordinarily much less than the errors of sampling. Rinse the contents of the bottle into a flask, titrate with $\frac{N}{10}$ solution of sodium thiosulphate using a few c.c. of the starch solution toward the end of the titration. Do not add the starch until the color has become a faint yellow; titrate until the blue color disappears.

Calculation of Results.—The standard method of expressing results shall be by parts per million of oxygen by weight.

It is sometimes convenient to know the number of c.c. of the gas per liter at 0° C. temperature and 760 mm. pressure and also to know the percentage which the amount of gas present is of the maximum amount capable of being dissolved by distilled water at the same temperature and pressure. All three methods of calculation are therefore here given.

$$\begin{aligned}\text{Oxygen in parts per million} &= \frac{0.0002N \times 1,000,000}{V} = \frac{200N}{V} \\ \text{Oxygen in c.c. per liter} &= \frac{0.1395N \times 1000}{V} = \frac{139.5N}{V} \\ \text{Oxygen in per cent of saturation} &= \frac{200N \times 100}{V \times O} = \frac{20000N}{VO}\end{aligned}$$

Where N = number of c.c. of $\frac{N}{10}$ thiosulphate solution.

V = capacity of the bottle in c.c. less the volume of the manganous sulphate and potassium iodide solution added (i. e., less four c.c.).

O = the amount of oxygen in parts per million in water saturated at the same temperature and pressure. See Table 13.

TABLE 13.
QUANTITIES OF DISSOLVED OXYGEN IN PARTS PER MILLION
BY WEIGHT IN WATER SATURATED WITH AIR AT
THE TEMPERATURE GIVEN.⁸⁶

Temp. C.	Oxygen	Temp. C.	Oxygen
0	14.70	16	9.94
1	14.28	17	9.75
2	13.88	18	9.56
3	13.50	19	9.37
4	13.14	20	9.19
5	12.80	21	9.01
6	12.47	22	8.84
7	12.16	23	8.67
8	11.86	24	8.51
9	11.58	25	8.35
10	11.31	26	8.19
11	11.05	27	8.03
12	10.80	28	7.88
13	10.57	29	7.74
14	10.35	30	7.60
15	10.14		

DETERMINATION OF FATS.⁸⁷

Evaporate 500 c.c. of sewage or other liquid in a porcelain evaporating dish to a volume of about 50 c.c. By means of a rubber-tipped glass rod remove to the bottom of the dish the solid matter attached to the sides, and add normal sulphuric acid to neutralize the alkalinity (with lacmoid). Do not use an excess of acid. Then evaporate the contents of the dish to dryness.

Treat the dry residue with boiling ether, rubbing the bottom and sides of the dish to insure complete solution of fat. Three extractions with ether are required.

Filter the ether solution through a five cm. filter into a weighed flask having a wide mouth. Evaporate the ether slowly, and put the flask in a desiccator for four hours or more. The increase in weight of the flask gives the amount of fats, or, in more precise language, the ether soluble matter.

Avoid an excess of acid, as it gives too high results due to acid fat residues.

DETERMINATION OF PUTRESCIBILITY.⁸⁸

This test, sometimes called the "incubator test," is of fairly recent English origin. It is now quite generally used in connection with sewage works analyses. Its purpose is to ascertain whether or not the quantity of organic matter in a sewage effluent

of an unstable or putrescible character is in excess of that which can be oxidized by the oxygen which it contains in the form of dissolved oxygen or oxygen available from nitrates, nitrites, and perhaps sulphates. While it is a very useful and important test in connection with sewage purification the object of which is the elimination of gross nuisances, and in studying the details of a highly polluted stream, it is a test which obviously has no direct bearing in the regular field of water analysis, or even in the purification of sewage the effluent of which without further treatment is intended to enter a stream used a short distance below for drinking purposes.

Procedure.—A round, glass-stoppered bottle of good quality having a capacity of at least four ounces is completely filled with the sample, and after being tightly stoppered is placed in an incubator at 37° C. As the sample is collected determinations are made of the dissolved oxygen, nitrogen as nitrites and nitrates, and the oxygen consumed by digestion in an acid solution with potassium permanganate at the room temperature for a period of three minutes. After the sample has been incubated 24 hours (or more), observations are carefully made as to the appearance of the sample, that is, whether it has turned black or not, and particular attention is given to the presence or absence of well-defined odors of putrefaction. Sometimes a qualitative test for the presence of sulphuretted hydrogen may be made with advantage, by suspending in the mouth of the bottle a strip of filter paper saturated with lead acetate. Some make this test⁸⁹ quantitatively.

Samples which after incubation are black in appearance, due to ferrous sulphide, and which possess foul odors, may be unquestionably regarded as putrescible without making any further tests.

Samples which at the end of the incubation period still contain an appreciable quantity of dissolved oxygen, or oxygen available from nitrates, and are free from sulphuretted hydrogen or other odors resulting from putrefaction, may be generally regarded with safety as non-putrescible.

Samples in which dissolved oxygen and nitrogen in the form of nitrates are absent, or nearly so, with more or less nitrogen in the form of nitrites, and in which the oxygen consumed when

determined for three minutes in the cold has increased on incubation, require more careful consideration before recording definitely the result of the putrescibility test. The best procedures by which any additional information can be obtained appears to vary under different local conditions as to character of sewage treated, the method of treatment, season of the year, etc., and it seems inadvisable now to specify in precise terms further procedures for use under all circumstances.

As the applicability of this test is studied in various laboratories, it is recommended that reports set forth distinctly the procedures by which conclusions have been arrived at with reference to putrescibility.

It is to be noted that this test is a very rigid one for practical conditions, inasmuch as sewage effluents almost invariably are diluted more or less by the oxygen-containing waters of the streams which they enter. In some laboratories the practice is adopted of making the tests as above outlined and also repeating the same process with samples diluted with equal or varying volumes of river water. Where facilities permit, the adoption of this test also on a diluted sample is to be recommended.

The results shall be recorded simply as positive or negative, stating clearly whether the sample was diluted, and if so, to what degree.

Note.—In regard to the incubation temperature of 37° C., as compared with 27° C. as used in England, the committee is aware of the fact that the effluents of coarse-grained sewage filters contain at times more dissolved oxygen than can be retained without pressure at 37° C. By using round bottles of high grade, with well-fitting glass stoppers, it does not appear that any serious amount of dissolved oxygen is lost by using this temperature. Cork stoppers and bottles having a poor quality of glass, especially square bottles, which break easily, are not permissible. In England the bottles prepared for incubation are sometimes "jointed," that is, provided with a mercury seal. As yet this procedure has received very little if any attention in this country.

The evidence now available indicates that the numbers and kinds of bacteria are sufficient in the effluents of coarse-grained filters to preclude any thought that the use of blood temperature for incubation interferes with the reliability of this test as compared with its application at lower temperatures.

In regard to the use of a 24-hour period of incubation, available evidence indicates that this is sufficient as a general proposition, although it is true

that now and then the result would be modified if the period of incubation were extended. Such modifications do not affect those samples which are either clearly putrescible or clearly non-putrescible, but refer to those samples which are very close to one side or the other of the dividing line between putrescibility and non-putrescibility. It is prudent to treat conservatively this intermediate class last mentioned until further data are available to supplement the procedures above given.

MICROSCOPICAL EXAMINATION.⁸⁰

The chief object of the microscopical examination of water is the determination of the presence or absence of those organisms which produce objectionable tastes and odors. In certain cases the examination is also of value as an index of pollutions, or as a guide to the identity of a water, i. e., whether surface water or ground water.

The term "microscopic organisms" shall be considered as comprising the diatomaceae, chlorophyceae, cyanophyceae, fungi, protozoa, rotifera, crustacea and other organisms microscopic in size, but not including the bacteria. Fragments of organic matter, broken down organisms, zoöglaea, etc., shall be termed amorphous matter. Clay, silt, oxide of iron and, in general, mineral matter shall not be included under amorphous matter, and shall not be measured by microscopic examination.

Special Apparatus.—1. A cylindrical funnel, about two inches in diameter, which has a capacity of about 500 c.c. and which terminates at the bottom in a tube about one-half inch in diameter, provided at the bottom with a perforated rubber stopper and a disk of silk bolting cloth just large enough to cover the hole.

2. A counting-cell consisting of a glass slide with a rectangular brass rim cemented on it, covered with a thin cover-glass; length of the cell, 50 mm., width, 20 mm., depth, 1 mm., capacity, 1 c.c.

3. An ocular micrometer consisting of a ruled square of such a size that, with the lenses used, it will cover an area of one square millimeter on the stage of the microscope. For convenience, this millimeter square is often subdivided into smaller squares.

Procedure.—Filter 250 c.c. of the water (more or less as the character of the sample demands) through a one-half inch layer of quartz sand (washed and screened between 60 and 100 mesh

sieves) placed at the bottom of the cylindrical funnel and supported by the rubber stopper and disk of bolting cloth. After filtration transfer the sand and the matter collected on it to a test tube, add 5 or 10 c.c. of distilled or filtered water and shake thoroughly. Pour off the liquid into a second tube. Put one c.c. of this concentrated sample into the cell, cover with a cover-glass, and allow the preparation to settle. Examine with a microscope, using a one-half inch objective and the micrometer above mentioned; count the organisms found in 20 squares taken at random in different parts of the cell. Knowing the degree of concentration and the number of squares counted, calculate the total number of organisms and express the results in number of standard units per c.c. One standard unit shall be considered as equal to a superficial area of 400 square microns. (One micron equals .001 millimeter.)

Note.—Inasmuch as many microscopic organisms are so fragile that they are liable to disintegrate during the process of filtration as above described, the regular procedure should always be supplemented by an ocular inspection of the original sample, followed, if necessary, by direct examination of the organisms. For the same reason the concentrated sample should not be allowed to stand long before the examination is made. Those microscopic organisms which are lighter than water rise to the top of the cell while the heavier organisms sink to the bottom. Care should be taken, therefore, in making the examination, to observe the entire depth of the liquid in the cell. If the cell is allowed to stand in front of a window some of the organisms may tend to move towards or away from the light and thus effect an unequal distribution of the contents of the cell. After counting the requisite number of cells it is therefore advisable to scrutinize the margins of the cell to see that no important organisms have been omitted from the record.

Expression of Results.—The results shall be expressed in whole numbers per c.c. The general directions as to significant figures given under Turbidity apply also to the microscopical examination.

BACTERIOLOGICAL EXAMINATION.

QUANTITATIVE BACTERIOLOGICAL DETERMINATIONS.⁹¹

In the present state of bacteriology there is no method known by which the absolute number of living bacteria in a sample of water can be determined, and all quantitative determinations of

bacteria are necessarily of a relative character. This being the case, strict adherence to a standard procedure⁷² is of especial importance.

Single isolated determinations of the number of bacteria in a surface water are of little value, but have more weight when accompanied by a full knowledge of the conditions under which the sample was collected, since rainfall, stream-flow, wind and many other factors materially influence the number of organisms present. A single examination may therefore readily lead to erroneous interpretation. Sometimes, however, it may afford some evidence as to the sanitary character of water, and scattered determinations are often useful in showing the relative character at different times of water obtained from any particular source. Quantitative bacterial determinations are of especial value as affording the best index of the efficiency of filtration. Here each separate test is of some importance.

Before the standard mode of procedure described below was recommended, the relative advantages of many methods were carefully considered from all points of view and submitted to practical comparative tests. The current practice of the leading water laboratories was also ascertained by means of a circular letter, the answers to which were set forth at length in the Second Report of Progress of this Committee, made in 1901.⁷³

Media.—The standard medium for determining the number of bacteria in water shall be nutrient gelatin, as is the case in Germany⁷⁴ and in England.⁷⁵ For field work, and for sewage and polluted waters which cannot be plated promptly after collection, agar may be substituted. All variations from these two media shall be considered as special media. If any medium other than standard gelatin is used, this fact shall be stated in the report.

For general work the standard reaction⁷⁷ shall be + 1 per cent, but for long continued work upon water from the same source the optimum reaction shall be ascertained by experiment and thereafter adhered to. If the reaction used, however, is different from the standard, it shall be so stated in the report.

The media shall be prepared as specified on pp. 104–110.

The use of simpler media,⁹⁸ such as albumose and agar dissolved in distilled water,⁹⁹ is a step in the right direction, but the evidence as to comparable results in various laboratories is still uncertain.

Procedure.—Shake at least 25 times the bottle which contains the sample. Withdraw one c.c. of the sample with a sterilized pipette and deliver it into a sterilized Petri dish, 10 cm. in diameter. If there is reason to suspect that the number of bacteria is more than 200 per c.c., mix one c.c. of the sample with nine c.c. of sterilized tap or distilled water. Shake 25 times and measure one c.c. of the diluted sample into a Petri dish. If a higher dilution is required proceed in the same manner, e. g., one c.c. of the sample to 99 c.c. of sterilized water, or one c.c. of the once diluted sample to nine c.c. of sterilized water, and so on. In the case of an unknown water or a sewage it is advisable to use several different dilutions for the same sample. To the liquid in the Petri dish add 10 c.c. of standard gelatin at a temperature of about 30° C., or 10 c.c. of standard agar at a temperature of about 40° C. Mix the medium and water thoroughly by tipping the dish back and forth, and spread the contents equally over the bottom of the plate. Allow the gelatin to cool rapidly on a horizontal surface and transfer to the 20° C. incubator as soon as it is hard. Incubate the culture for 48 hours¹⁰⁰ at a temperature of 20° C.¹⁰¹ in a dark, well-ventilated incubator where the atmosphere is practically saturated with moisture.¹⁰² After this period of incubation place the Petri dish on a glass plate suitably ruled and count the colonies with the aid of a lens which magnifies at least five diameters. So far as practicable the number of colonies upon the plate shall not be allowed to exceed 200. The whole number of colonies upon the plate shall be counted, the practice of counting a fractional part being resorted to only in case of necessity.

When agar is used for plating it will be found advantageous to use Petri dishes with porous earthenware covers* in order to avoid the spreading of colonies by the water of condensation.¹⁰³

Expression of Results.—In order to avoid fictitious accuracy and yet express the numerical results by a method consistent with the precision of the work the rules given below¹⁰⁰ shall be followed:

* Made by Hews Pottery Co., Cambridgeport, Mass.

Numbers of Bacteria per c.c.

From	1 to	50	Recorded as found	
"	51	"	100	" to the nearest 5
"	101	"	250	" " " " 10
"	251	"	500	" " " " 25
"	501	"	1,000	" " " " 50
"	1,001	"	10,000	" " " " 100
"	10,001	"	50,000	" " " " 500
"	50,001	"	100,000	" " " " 1,000
"	100,001	"	500,000	" " " " 10,000
"	500,001	"	1,000,000	" " " " 50,000
"	1,000,001	"	10,000,000	" " " " 100,000

Note.—The determination of the number of bacteria which develop at 20° C. under anaërobic conditions, the number which develop at 37° C.,¹⁰⁴ the number of red colonies¹⁰⁶ which develop on a lactose litmus agar plate, and the number which develop on media other than the standard are not advised as regular procedures for either water or sewage. *B. coli* determinations are more valuable; species determinations may also be useful. No uniform methods of procedure for the special determinations above listed are here given, as the value of their determination depends upon the individuality of local conditions for each problem.

TEST FOR BACILLUS COLI.¹⁰⁶

During the past decade the test for *B. coli* has increased more and more in importance. This organism is identical with, or at least very closely allied to, bacteria which are found somewhat widely distributed in nature,¹⁰⁷ and is essentially an organism whose habitat is the intestines of man¹⁰⁸ and of warm-blooded animals.¹⁰⁹ For this reason its presence in water is to some extent indicative of pollution, although its abundance rather than its mere presence must be considered as the criterion. The test for *B. coli*, in order to be of definite value, therefore, must be not only qualitative but quantitative.

Quantitative vs. Qualitative Results.—While colon bacilli in water cannot be determined quantitatively with such precision as can the mineral constituents, yet with sufficient care results may be obtained which are very valuable comparatively, and which are approximately quantitative. Qualitative results, when viewed superficially, may seem easier to obtain than quantitative and quite as conclusive; but detailed evidence shows that in general the quantitative tests are by far the more fruitful branch of study.

The committee therefore urges that more attention be given to this line of investigation, even if fewer samples are tested.

Diagnostic Characters.—The test for the presence of *B. coli* in water shall not be considered complete unless the organism is isolated in pure culture, in accordance with procedures described on pp. 90–91 and is found to show the following characters:

1. Typical morphology—non-sporing bacillus, relatively small and often quite thick.

Debilitated forms of colon bacilli sometimes show polar staining, giving the appearance of diplococcoid forms.¹¹⁰ Such cultures show general physiological debilitation, including loss of motility, of power to ferment carbohydrates, to produce indol, etc. These and other facts accentuate the desirability of employing for this test methods which provide for regeneration by adequate preliminary cultivation.¹¹¹ Warning is especially directed against recording a negative result for the test as a whole, when abnormal features in some particulars have been encountered, until reasonable efforts have been made to restore the normal characters of a culture. The diplococcoid form above mentioned is not to be confused with sewage streptococci,¹¹² a separate group of bacteria often characteristic of pollution.

2. Motility—when a young broth or gelatin culture is examined.

Sometimes even young cultures of the colon bacillus do not show motility; but in these cases the process of rejuvenation,¹¹¹ will usually restore the normal character.

3. Non-liquefaction of gelatin.

Occasionally it may happen that the gelatin stab cultures are not allowed time enough to effect liquefaction. One week at a temperature of about 20° C. is a minimum period; ten days is a much safer one; while some workers prefer a period of two weeks. Some forms of *B. cloacae* liquefy only after a still longer period.

4. Fermentation of dextrose broth, with the formation of about 50 per cent of gas, of which about one-third (CO_2) is absorbed by a two per cent solution of sodium hydrate.

Colon bacilli may give quite wide variations¹¹³ in the amount of gas produced by the fermentation of carbohydrates. The above stated quantities of total gas and of CO_2 (gas absorbed by sodium or potassium hydrate) are general averages. Deviations from these averages can usually be avoided by rejuvenation. In the fermentation of dextrose by colon bacilli the amount of gas formed often varies from 30 to 70 per cent, with a corresponding variation in the amount of CO_2 . The use of carbohydrates other than dextrose in the fermentation tube is occasionally,

but not generally, important here. Lactose, however, is coming more and more into use in testing the water sample, prior to obtaining the species in pure culture.

5. Coagulation of milk, with the production of acid, in 48 hours or more at 37° C., either spontaneously or upon boiling.

Cultures are usually made in milk to determine acid formation and coagulation; but recent studies¹¹⁴ indicate that such a procedure is unnecessary when a pure culture has already been tested for acid production in litmus lactose agar. Reddening of this medium invariably indicates that in milk the acid formation and coagulation will follow. Casein is not digested by *B. coli*. However, the committee advises that the use of milk be continued as a confirmatory test until further evidence is available upon the points just stated.

6. Production of indol in peptone solution.

Somewhat variable results with this reaction¹¹⁵ have cast doubt upon the advisability of its continuance as a diagnostic test. Such variations, however, can be largely overcome by improved methods, including preliminary cultivation.¹¹¹

7. Reduction of nitrates.

The statement regarding the indol test applies also here, though in a less degree.

Quantities of Water Tested.—For ordinary waters, 0.1, 1.0 and 10.0 c.c. shall be used for the colon test. For sewage and highly polluted surface waters, smaller quantities shall be used; and for ground waters, filtered waters, etc., the quantities shall be larger, if necessary to obtain positive results. The quantities shall vary preferably in the tenfold manner indicated. Single tests with quantities which give ordinarily a positive result or ordinarily a negative result are in themselves of but little account for quantitative determinations. The range in quantities studied shall be sufficient to allow the quantities needed for both a positive and a negative result to be recorded for each sample. When this is done, the results of several tests allow an approximate estimate of the number of *B. coli* per c.c.

Present Procedures for Treating Samples.—There are two methods in use by which water may be so tested as to obtain approximately quantitative determinations for colon bacilli. These may be termed, for convenience, A and B.

A. The preparation of an agar plate with a known volume of water, using a medium which contains a sugar and is colored blue with litmus. The plate is incubated at 40° C. Under these conditions *B. coli* and some other species show their presence by red colonies, due to acid fermentation of the carbohydrate. It must be remarked that not all red colonies are to be regarded as *B. coli* without further tests.

B. The preliminary cultivation at 40° C. of a known volume of water in a fermentation tube containing a sugar broth. If gas appears, a portion of the liquid is plated on agar, practically as in procedure A above, for the determination of *B. coli*. This procedure has quantitative value only when numerous portions of the water sample are tested to ascertain the approximate volume above which the results are positive and below which they are negative.

Each of these methods has some disadvantages, due in part to difficulties of technique. Both allow the use of only a very limited quantity of water, 5 to 10 c.c. at most; and the plate method, in testing polluted waters, necessitates the use of quantities of water so small that the red colonies are sufficiently separated to permit individual ones to be fished. Even when the plates are inverted, spreading colonies not only overgrow and obscure the red, but also render uncertain the question of pure cultures, and necessitate the labor of obtaining the cultures in purity after the colony has been fished. The use of Petri dishes with porous tops, however, largely overcomes this difficulty. As the method is commonly practiced, it has also the drawback of not affording a sufficient number of red colonies to serve as reliable data. In the fermentation tube method the disadvantages arising from overgrowths¹¹⁷ and from the antagonism of other species are also present,¹¹⁸ thus making somewhat difficult the task of obtaining later plate cultures of *B. coli*.*

*For the sake of explicitness the committee desires to call attention to the following definitions regarding growths of mixed cultures: Overgrowth means the predominance of one species over another as regards numbers of bacteria, but without necessarily any benign or prejudicial influence being exerted upon other species present. This term is also used with reference to extensive surface-film formation. Antagonism refers to the prejudicial influence of the growth of one species over another, while symbiosis involves a favorable influence of one species upon another.

To obviate these disadvantages, the following attempts at improvement in technique have been made, the discussion of which is pertinent here.

1. Concentration. As it is not possible by the procedures above described to treat directly more than 10 c.c. of a sample, and as filtered water (and some others) rarely contains *B. coli* in this volume, attention has been directed to the concentration of bacteria from relatively large quantities of water into volumes within reach of usual methods. This has been accomplished more or less satisfactorily in different ways, as follows:

a. By filtering through a sterilized filter tube of unglazed porcelain, and then removing the sediment by a sterilized filter brush with a small volume of sterilized liquid.

b. By means of centrifugalization.

c. By cultivating in a flask a mixture containing the desired volume of water with a suitable volume of sugar broth, and then if gas bubbles are noted, by treating some of the sediment in the usual way. Occasionally the sugar broths are made of increased strength so as to make the resulting mixture normal.

While these means of concentration have been helpful, they are not to be recommended, on account of the disturbances, due to overgrowth, etc., which they cause in securing quantitative data.

Where such waters are frequently studied it is advised that the fermentation tubes used be of such size as to allow at least 10 c.c. to be tested at a time, and that as many portions be tested as found necessary. It is obviously less expensive for laboratories connected with large water plants to obtain special apparatus for this purpose than to increase several fold the labor in getting reliable data.

2. Inhibiting Agents.—In addition to the use of a high temperature (the recent English report⁸⁶ strongly recommends incubation of the fermentation tube anaerobically at 42° C.), numerous efforts have been made to modify the composition of media so as to inhibit the growth of some of the bacteria commonly present in water and sewage. Attention has been given not only to various degrees of reaction, but to the addition of small quan-

tities of germicides, particularly phenol.¹¹⁸ Opinion differs materially as to the value of the latter, indicating its special and not general applicability, and suggesting further that there ought to be recorded the results of comparative studies to show what species of bacteria are inhibited by its use.

Sodium taurocholate¹¹⁹ (bile salt) is thought well of abroad⁸⁶ for inhibiting the growth of the more common spore-forming organisms, but in this country experience with it has been limited and uncertain.¹²⁰

Special Media for Colon Tests.—General directions are given on p. 104 *et seq.*, for the preparation of the media. It is desired here also to call attention to the care necessary to prevent inversion of the sugar in preparing carbohydrate media. Solutions of litmus and of sugars are prejudicially affected by high temperatures. For this reason the intermittent method of sterilization should be used, and not the continuous or autoclave method. It is still safer to sterilize the azolitmin (and perhaps the sugars) separately at a low temperature, and to add them by means of a sterilized pipette after the media have been sterilized.

From present evidence it appears advisable when working under some conditions to exchange the sugars as commonly used in agar plates¹²¹ and fermentation tubes.¹²² In the latter it is highly desirable to restrict gas formation by other organisms than *B. coli*, and to this end lactose is more suitable than glucose or dextrose. In the agar plates, however, there are good grounds for believing that the gas-formers should make themselves apparent to the fullest extent, thus requiring *B. coli* to be identified from among the gas producers rather than from among the acid producers, as is the custom at present.

Each lot of agar, with its litmus and carbohydrate, should be thoroughly tested with a culture of *B. coli* to make sure that the conditions for gas formation and acid formation are facilitated as far as practicable. Now that spreading colonies are so nearly eliminated by the porous covers, the practice of each laboratory should be reconsidered with regard to the above mentioned points.

Nutrose agar¹²³ is thought well of abroad⁸⁶ and also in this country,¹²⁰ although some modifications in its preparation have

been made. While on present evidence the committee hesitates in advising its general use, it commends the investigation of its merits under a wide range of conditions.

The use of neutral red^{12a} has been found practicable by some investigators as an aid in isolating the colon bacillus, but while with certain waters this may be so, wider experience has shown the general unreliability of the method; its use is therefore not advised.

Recommended Procedures for Treating Samples.—Having in mind the facts given above, the committee has added the following details to methods A and B for treating water samples.

Procedure A.—This method is most applicable for sewages and polluted waters in which *B. coli* is present in one c.c. or less. It involves the preparation of agar plates in the usual manner as to dilution etc. (cf. p. 83), and with especial attention to the use of carbohydrate and litmus as above directed. Petri dishes with porous covers shall be used.

Incubate the plates at 40° C.* for 12 to 24 hours. If no red colonies or gas-producing colonies appear, then *B. coli* is considered absent in the volume of the sample tested.

If colonies resembling *B. coli* are noted, then obtain a pure culture of each if the total number of such on the plate does not exceed five or six. If the number is larger, fish at least five colonies, but take them from an aliquot portion of the plate. The information afforded by a series of tests of the sample in fermentation tubes is frequently of sufficient aid in deciding upon the number of colonies to fish to make the preparation of these controls worth while.

As an optional adjunct to the ordinary plate method, cultures in purity may be obtained after a little practice by the use of streak plates. Nutrient agar is poured into Petri dishes and, if those provided with earthenware covers are not available the plates are allowed to remain in an inverted position, uninoculated, for 24 hours or so in order to obtain a relatively dry surface.

* The committee advises that where practicable an incubator at 40° C. be provided for *B. coli* work; where a special incubator for *B. coli* work is not feasible the regular 37° C. incubator may be used, but a record should be made in each case showing the actual temperature used.

Inoculation is performed by drawing the needle several times across the surface of the agar without recharging it with inoculable material.

If the colonies obtained in pure culture resemble *B. coli*, then make sub-cultures upon a slanted agar tube (to preserve the culture for future study and reference), in fermentation tubes, milk, gelatin tube, peptone solution (for indol), and nitrate broth.

After examining the sub-culture, record the final result in accordance with the standard diagnostic characters stated on pages 85-86.

Procedure B.—This method is most applicable for waters requiring one or more c.c. to be tested for a positive result. It is also useful as a control for Procedure A, as just stated.

Transfer a measured quantity of the sample to two (or preferably more) lactose or dextrose fermentation tubes in accordance with the general directions of the foregoing pages.

Incubate the tubes at 40° C. for 48 hours, noting the presence of gas, if any, twice a day.

If no gas forms, record the result as negative.

As soon gas as has formed (usually in about 16-24 hours) plate at once in lactose litmus agar a portion of the sediment at the bottom of the closed arm, and continue the test as given for Procedure A.

It is necessary to note that while a method satisfactory in one laboratory for a given set of conditions can be used elsewhere for similar conditions, it by no means follows that such a method will be universally applicable. In fact, it is known that different conditions call for different details of procedure. Uniformity in this case must consist not in a blind adoption of a given technique, but in an adjustment of the underlying principles of the method to fit the conditions at hand.

PRESUMPTIVE TESTS.¹²⁶

Partial tests for *B. coli* by which several but not all of the foregoing characteristics of the organism are ascertained are considered useful under some circumstances, especially where the

time allowed for making the test is necessarily limited. As a general rule, however, where tests for *B. coli* are needed it is believed that it is unwise to use methods giving less definite results than those described above.

TESTS FOR SEWAGE STREPTOCOCCI.

Some English bacteriologists are inclined to regard streptococci as indicating recent and objectionable pollution. In some cases it may be of value to test the sample of water for streptococci, but the information afforded by the occurrence of these organisms seems to be of less value than in the case of *B. coli* and the committee believes that, for the present at least, the streptococcus test is of subordinate importance.

DETERMINATION OF SPECIES OF BACTERIA.*¹²⁷

The determination of all the species of bacteria present in a sample of water or sewage is seldom practicable and the results of such determinations as are usually made, are at present of little direct value. This phase of water analysis is likely, however, to develop in the future and may eventually come to have greater importance.

Lack of uniformity, and indefiniteness¹²⁸ in description rendered a great part of the early work on bacterial species utterly valueless. It was this fact, indeed, that gave rise to the present movement for standard methods. The report of the Bacteriological Committee of 1897 has been a potent factor in this country in raising the standard of determinative bacteriology. The advantage of standard methods was practically illustrated by the co-operative work recently carried on by this committee and described in the appendix to this report.

In certain cases the determination of species may play directly a useful part in the analyses of water. It sometimes happens that the bacteria present in a filtered water are different in character¹²⁹ from those found in the raw water and unless the facts are known erroneous inferences may be drawn as to the efficiency of the filters. The determination of particular species is sometimes of importance in proving the identity of water from some particular source. At times also the presence of certain species in water may be indicative of pollution.

In the present state of our knowledge it is believed that information concerning the presence of streptococci, of *B. aërogenes capsulatus* (*B. enteritidis sporogenes*), of liquefying bacteria, etc., is not of sufficient value to warrant carrying out regular tests for these organisms. The determination of the presence of *B. coli*, however, is of such significance from the sanitary standpoint that a separate section of the report is devoted to that subject.

The occurrence of the typhoid bacillus and other pathogenic intestinal organisms is of course a matter of supreme importance, and, although it is usually impracticable to determine their

* Revision of the 1897 Report.⁴

presence in water or sewage, except when they are very abundant, the question of selective methods is a vital one.

If the identification of various species of bacteria could be more easily accomplished and rendered quantitative within reasonable limits the results obtained by studying some of the more common and harmless bacteria would doubtless prove highly profitable. With this object in view the prescribed determinations which follow have been formulated so as to simplify these methods as much as possible and at the same time have them consistent with the accumulation of the data necessary for accurate identification.

The methods given are those especially applicable to the bacteria ordinarily found in water and sewage. They may be applied also to those bacteria that are involved in industrial processes. As applied to pathogenic bacteria, however, and to those species associated with the human body, they may prove somewhat deficient, but it is believed that even in the field of medical bacteriology the methods described will serve a useful purpose by systematizing results, and co-ordinating the work done by bacteriologists working in different fields.

Some of the biological tests described in the 1897 report of the Bacteriological Committee are here omitted. This is because they have not been found to be generally practicable, that is, the results are either not commensurate with the labor involved, or they are so variable under certain conditions as to have no diagnostic value.

It is becoming evident that in the future more detailed attention must be given to morphology and that the biochemical reactions must be made more precise. The hair-splitting methods of differentiation on the basis of arbitrarily selected cultural characteristics which were so common in the earlier days of the science must give way to more rational and broader ideas respecting the distinguishing marks of certain groups of bacteria, and it must also be recognized that these minute organisms are profoundly influenced by environmental conditions.

In the 1897 report the tests of bacteria were divided into two

groups,—necessary tests and optional tests. Practical experience has shown that most of the tests characterized as optional are of comparatively little importance in the study of water bacteria and that their use is extremely limited. The same may be said with respect to a few of the criteria formerly considered necessary, as, for example, the cultural characteristics on potato and blood serum. Of the necessary tests described in the former report only those have been retained which are considered to be of greatest importance. No doubt in particular cases some of the optional tests may yield data highly desirable and very instructive. These tests are described in the standard text books on bacteriology, and need not be detailed here.

In studying pathogenic bacteria found in water it is necessary to secure data in regard to the pathogenesis of the organism and to make studies regarding agglutination, etc. These are matters more particularly allied to medical bacteriology and while the need of their occasional use is recognized it is believed that they do not properly come within the sphere of this report.

The tests here recommended for differentiating species of bacteria may be divided for convenience into two groups—primary tests and supplementary tests. The primary tests are those which are of chief importance and which are necessary for differentiation in the majority of cases. They include those tests in which a definite positive or negative result may be obtained, thus enabling them to serve as a basis of classification. The supplementary data are ordinarily, but not always, of less importance than the primary tests. Sometimes they are highly desirable and nearly always instructive. It is recognized that this difference is an arbitrary and a provisional one, destined in time to give way when a more comprehensive system of taxonomy shall be developed; but it is believed that it fairly represents the present development of differential bacteriology and that it will serve for the immediate future.

The following data are required for the determination of bacterial species, and may be considered as representing the necessary minimum. Those which are classed as primary tests are indicated under Expression of Results (see p. 118).

- I. Source and habitat.
- II. Morphological characters.
 - Form.
 - Manner of grouping.
 - Dimensions.
 - Staining reactions:
 - a. with watery dyes; b. by Gram's method.
 - Presence or absence of flagella (motility).
 - Presence of spores and their character.
 - Fission.
 - Capsules.
 - Involution and degeneration forms.
- III. Cultural characteristics, mode of growth in and upon:
 - Nutrient broth.
 - Gelatin plates.
 - Gelatin tubes.
 - Agar plates.
 - Agar tubes.
- IV. Biochemical reactions.
 - Action upon milk (reaction and digestion of casein).
 - Action upon carbohydrates (fermentation, gas formation, production of acidity, etc).
 - Action upon nitrates.
 - Production of indol.
 - Inhibition of growth by acidity and alkalinity of media.
 - Relation to free oxygen (aërobic and anaërobic growth).
 - Temperature relations (activity of growth at 20° C. and at 37° C. and thermal death point).
 - Pigment formation.
 - Liquefaction of gelatin.

SOURCE AND HABITAT.

No uniform method or statement of facts regarding sources and habitat is possible. The data, however, shall be given with as much fullness as the case demands in order to obtain information for estimating the possible influence of environment.

MORPHOLOGICAL CHARACTERS.¹²⁰

The morphological characters shall be determined and described from growths obtained upon at least one solid medium (nutrient agar), and in at least one liquid medium (nutrient broth). Growths at 37° C. shall be in general not older than 24 to 48 hours, and growths at 20° C. not older than 48 to 72 hours, yet it is important that the cultures be fully developed, as those which are too young may present immature forms due to rapid multiplication, while those which are old may contain altered or

degenerated forms. The growth from both solid and liquid media shall be examined both in stained preparations and in hanging blocks or hanging drops. Morphological descriptions shall be always accompanied by definite statements regarding the medium used and the temperature and age of the culture. It is important to examine the cultures both stained and unstained.

Form and Grouping.

Determine the form and grouping from growths on solid and in liquid media as stated in the foregoing paragraph.

Procedure for Hanging Block.¹⁸¹

Pour melted nutrient agar into a Petri dish to the depth of about one-eighth to one-quarter inch. Cool this agar and cut from it a block about one-quarter inch to one-third inch square and of the thickness of the agar layer in the dish. This block has a smooth upper and under surface. Place it, under surface down, on a slide and protect it from dust. Prepare in sterile water an emulsion of the organism to be examined, if it has been grown on a solid medium, or use a broth culture; spread the emulsion or broth upon the upper surface of the block as if making an ordinary coverslip preparation. Place the slide and block in a 37° C. incubator for 5 or 10 minutes to dry slightly. Then lay a clean sterile coverslip on the inoculated surface of the block in close contact with it, as far as possible avoiding air-bubbles. Remove the slide from the lower surface of the block and invert the coverslip so that the agar block is uppermost. With a platinum loop run a drop or two of melted agar along each side of the agar block, to fill the angles between the sides of the block and the coverslip. This seal hardens at once, preventing slipping of the block. Place the preparation in the incubator again for 5 or 10 minutes to dry the agar seal. Invert this preparation over a moist chamber and seal the coverslip in place with white wax or paraffin. Vaseline softens too readily at 37° C. allowing shifting of the coverslip. The preparation may then be examined as desired with a $\frac{1}{12}$ inch homogeneous immersion objective.

Procedure for Hanging Drop.

Place a drop of fluid medium (or 0.85 per cent NaCl sol.) on a sterile cover-glass and inoculate the edge with a minute portion of the culture. Invert this preparation over a hollow slide, seal with vaseline and examine with a $\frac{1}{4}$ inch homogeneous immersion objective.

Impression preparations of surface colonies are sometimes valuable. Determine the form also from stained preparations.

Until the labors of Migula, Fischer and others, who are seeking to place the classification of bacteria on a more scientific and substantial basis, shall have borne fruit, the old classification will be adhered to, namely :

Coccus, or Micrococcus.—(Forms which are spherical, or nearly so).

Single coccus, grouped irregularly.

Diplococcus, forming pairs.

Streptococcus, forming chains, often showing paired cocci.

Tetracoccus, forming fours by division through two planes.

Sarcina, forming packets of eight, or more, members, by division through three planes.

Bacillus.—(Cylindrical forms having one dimension decidedly greater than another, more or less straight and never forming spirals.)

Single bacillus.

Diplo- and strepto-bacillus, forming pairs and longer chains, the bacilli being attached end to end.

Filaments, or thread-like growths, in which divisions into bacilli of the normal length are not apparent, or occur irregularly and transversely to the long axis of the growth.

Spirillum.—Curved or twisted forms, constituting complete spirals or portions of spirals.

Dimensions.

Determine the dimensions of the bacterial cell both in stained preparations from agar cultures (made preferably with aqueous gentian violet), and in unstained hanging block or hanging drop preparations.

The most accurate way of determining the size of bacteria is by photography, but where this method cannot be used the measurements may be made by an ocular micrometer. To insure accuracy and uniformity measure the individuals just after fission has been observed and before a new fission takes place. Determine

the width, the average length and the extreme length. State the dimensions in microns and decimals of a micron (1 micron equals .001 mm.).

Staining.

The primary object of staining bacteria is to make them more easily observed under the microscope with reference to their general morphology, but it has been found that different species react differently to similar stains, so that to some extent staining has a diagnostic value. The general process of staining is as follows :

Reagents.—1. An aqueous solution of fuchsin made by adding 5 c.c. of a saturated alcoholic solution of basic fuchsin to 95 c.c. of distilled water.

2. An aqueous solution of methylene blue similarly prepared.

3. An aqueous solution of gentian violet similarly prepared.

4. Alkaline solution of methylene blue :

Saturated alcoholic solution of methylene blue	-	30 c.c.
Potassium hydrate, one per cent solution	-	1 c.c.
Distilled water	- - - - -	100 c.c.

5. Carbol fuchsin :

Basic fuchsin	- - - - -	1 g.
Alcohol	- - - - -	10 c.c.
Carbolic acid	- - - - -	5 g.
Distilled water	- - - - -	100 c.c.

Or this may be prepared by adding to a five per cent aqueous solution of carbolic acid enough saturated alcoholic solution of fuchsin to produce a metallic luster on the surface of the fluid. Filter before using.

6. Aniline water gentian violet. To 98 c.c. of distilled water add 2 c.c. aniline oil, shaking until the fluid becomes clear. Then filter and to 75 c.c. add the aniline water 25 c.c. saturated alcoholic solution of gentian violet. This solution will keep for two to three months if not exposed to strong light.

7. Iodine solution (Gram's):

Iodine	- - - - -	1 g.
Potassium iodide	- - - - -	2 g.
Distilled water	- - - - -	300 c.c.

Procedure.—Clean a cover-glass,* removing the organic matter by immersing in a solution of potassium bichromate in dilute sulphuric acid, afterwards washing thoroughly. On the cover-glass make a very dilute emulsion of the culture in clean sterile tap water. Spread this as a thin film over the cover-glass and dry rapidly in the air. For ordinary purposes of observation fix the film by rapidly passing the cover-glass three times through a flame, but in the case of specimens to be used for measurement fix the film by heating in an automatically regulated air bath, taking care to keep the cover-glass from direct contact with the shelf of the oven. The difficulty of fixing preparations from liquid media may be overcome by allowing a few drops of 95 per cent alcohol to evaporate from the hot smear during the ordinary fixing processes, or by previously centrifugalizing the liquid culture. The latter method has the advantage of removing the debris of the medium from the smear. At least three stained preparations are required in all cases, namely: (1), made by using a watery dye; (2), by using aniline gentian violet; and (3), by using Gram's stain. Stain the preparation without heating; wash with clean water; mount in water or in balsam and examine under a magnifying power not less than that given by a $\frac{1}{1\frac{1}{2}}$ inch homogeneous immersion lens and a No. 3 Huygenian eye-piece.

Inasmuch as media of different refractive indices make differences in the picture obtained and inasmuch as no one medium is suitable for all cases, the kind of medium used shall be stated in all descriptions. The media most commonly used for mounting are water, balsam dissolved in xylol, or cedar oil, the former having a low index of refraction, the latter a high index. Balsam is recommended for general use.

In observing the stained preparations record the form and grouping, make the necessary measurements, note the internal structure of the organism as shown in both faintly and deeply stained specimens, and also the readiness with which the organism takes the stain. Make particular note of the presence of

* For routine work the committee does not discourage the usual laboratory practice of making film preparations on slides instead of on coverslips.

vacuoles, capsules, spores, flagella, etc. These determinations in some cases require special modes of staining.

Procedure of Staining by Gram's Method.—Prepare and fix the preparation as above described and stain for one minute with aniline gentian violet. Wash the preparation and immerse for two minutes in Gram's iodine solution. Rinse in 95 per cent alcohol for five minutes; dry; mount in balsam and examine.

In determining the comparative staining capacities of different species, especially by Gram's method, it will sometimes be found convenient to mix the species under examination with two other species (e.g. *B. typhosus* and *B. diphtheriae*), one of which does and the other does not stain by the method used.

Use of Acid Decolorizers.—The application of acid decolorizers to stained specimens is sometimes of value in distinguishing bacterial species. Descriptions of these processes may be found in standard text books.

*Flagella.*¹³²

No single method is equally applicable to all cases but the following have been widely and successfully used:

Method 1. (van Ermengem's).

Reagents.—1. Solution A (fixative bath)—

Osmic acid, 2 per cent aqueous solution	-	-	-	-	1 part
Tannin, 10 to 25 per cent aqueous solution	-	-	-	-	2 parts

2. Solution B (sensitizing bath)—

Nitrate of silver, one-half per cent aqueous solution.

3. Solution C (reducing and reinforcing bath)—

Gallic acid	-	-	-	-	-	-	-	5 grams
Tannin	-	-	-	-	-	-	-	3 grams
Potassium acetate (fused)	-	-	-	-	-	-	-	10 grams
Distilled water	-	-	-	-	-	-	-	350 grams

Procedure.—Place the films in solution A for one hour at room temperature, or in a watch glass over a water-bath at 100° C. for five minutes. Wash with distilled water, then with absolute alcohol for three or four minutes, and again in distilled water. Transfer films to solution B contained in a shallow vessel, and agitate them for five seconds; then without washing transfer them to solution C in a similar dish, for five seconds with continuous

agitation, repeating baths B and C in rotation until specimens turn black-brown. It is now advisable to mount them in water and examine with a high power dry lens to ascertain the degree of staining acquired by the flagella; if not dark enough repeat baths B and C for a few times more, wash in distilled water, dry, and mount in balsam. It is advisable to renew baths B and C as soon as any deposit in them is noticeable.

Method 2. (Bunge's).

Reagents.—1. Mordant.

Five per cent aqueous solution of ferric chloride	-	-	-	1 part
Saturated aqueous solution of tannic acid	-	-	-	3 parts

2. Carbol-fuchsin.

Procedure.—Prepare a fresh agar streak culture. Remove some of the culture, being careful to avoid taking the culture medium, and add this to sterile tap (not distilled) water. Allow this to stand and diffuse rather than distribute it by stirring, thus preventing breaking the flagella. Should a liquid culture be used, centrifugalize it before withdrawing a portion of it for testing. It may be necessary to centrifugalize and decant several times before clearing the medium of all debris. Place some of the preparation on a scrupulously clean cover-glass and fix the film, taking great care to avoid overheating, as this may ruin the preparation. Treat over steam of a water bath with the mordant for two minutes; wash; and stain two minutes over water bath with carbol-fuchsin; wash; dry; and mount in balsam.

Describe the number and character of the flagella, noting particularly their location; in some of the new systems of classification these matters have a high diagnostic value.

Spores.

No process for staining spores is the best in all cases, but the following generally gives satisfactory results:

Procedure.—Prepare a cover-glass preparation from a 48-hour old culture. Stain by the usual methods and note the presence of spores, describing their size, their relation to the mother-cell and their location in the same. Note also the presence of free spores in the culture and determine their size and shape, whether

spherical, oval, elliptical, oblong, etc. If endospores are present they may be stained as follows:

Moeller's Method.—Place the films in chloroform for two minutes; then in a five per cent aqueous solution of chromic acid for two minutes; wash in water; cover with carbol-fuchsin and heat in the steam of a water-bath for five minutes; wash excess of stain off in water; decolorize not too vigorously in one per cent sulphuric acid; wash in water; counterstain with alkaline methylene blue for 10 seconds at room temperature.

The manner of germination of spores is of taxonomic importance and shall be studied by direct observation by the hanging block method.

Note.—In examining bacteria for the presence of spores care must be taken not to mistake for spores the small bright refulgent areas which are often seen and which usually do not take up the dye. In some cases these appearances are caused by vacuoles, fat drops or crystals. The only method of identification of spores which is absolutely positive is that of observing germination.

An approximate method of determining the presence of spores is to test the resistance of the suspected bacteria to moist heat at a temperature of 80° C. The procedure for this is described under "Temperature Relations." According to this method the presence of spores is indicated by the ability of the culture to develop after being subjected to a temperature of 80° C. for 15 minutes.

*Fission.*¹³³

The recent observation that bacterial fission follows at least two distinct types, the ordinary method of division and that known as "snapping," the latter being a characteristic of the diphtheroid group, makes it advisable to examine all new species by direct microscopic observation in order to observe individuals undergoing multiplication. The hanging block method is well adapted to this purpose.

Capsules.

As the production of capsules is often dependent upon the presence of albumin in the culture medium the organism to be tested for capsules should be grown in milk or in serum media.¹⁴⁴

Procedure.—Prepare the film from the medium without the use of water; fix; apply glacial acetic acid; drain immediately without washing; treat with the stain and examine in dilute salt solution.

Involution Forms.

Involution changes can be most readily recognized in stained preparations. The condition of both young and old cultures should be noted. Degenerate appearances are more likely to be observed in the latter; but in some cases they are found even in vigorous cultures.

CULTURAL CHARACTERS.

Certain biological characters of bacteria are determined by cultivating the organisms in or upon various culture media and, after a certain period of incubation, noting, first, the mass characteristics of the organisms in the culture and, second, the biochemical reactions which they produce. Experience has shown that it is difficult to obtain consistent results upon these points because the nature of the growth is influenced by many conditions, such as the original vitality of the bacteria themselves, the composition and concentration of the culture medium, the period and temperature of incubation, the amount of moisture in the atmosphere, etc. Within limits, however, it may be anticipated that the more nearly these conditions can be reduced to uniform practice the more reliable will be the comparisons made between different investigations.

PREPARATION OF CULTURE MEDIA.¹²⁴

In view of the marked influence upon bacteriological reactions of variations in culture media caused by differences both in ingredients and in technique of preparation, it is necessary that uniform methods be used in order to obtain comparable data. In specifying the various ingredients used in culture media it is the intention of the committee that they shall be uniform in quality, but it is not the intention that the recommendations as to ingredients and technical manipulations shall stand in the way of true progress as to improvements. When, however, improved or modified methods are used, the variations from the standard methods shall be plainly set forth together with the reasons for the modifications.

Ingredients.

Distilled water shall be used in the preparation of standard culture media.

Infusions of fresh lean meat, and not meat extract, shall be used as the basis of various media.

Sodium chloride shall not be added to any culture medium herein specified.

Peptone shall be that of Witte (dry from meat).

Gelatin shall be the best French brand, so-called. It shall be as free as possible from acids and other impurities, and shall be of such a character that a 10 per cent solution prepared in the usual way shall not soften when kept at a temperature of 25° C.

Commercial agar in threads shall be of as high a grade as can be obtained. Agar may be purified by washing.

The various sugars such as dextrose, lactose and saccharose, shall be as nearly as possible the chemically pure compounds designated. Unusual effort to obtain such sugars is considered to be necessary.

Glycerin shall be double distilled.

In place of litmus azolitmin shall be used as a one per cent aqueous solution.

Of the various other ingredients used, nearly all of which are of a mineral nature, special effort shall be made to see that they are chemically pure products within the full meaning of this expression.

Sterilization.

Of the two available methods of sterilization, the intermittent method at a temperature of 100° C. is considered on the whole to be preferable. The higher temperatures of the autoclave facilitate chemical reactions and changes which in some cases are undesirable.

When the latter method is used media contained in ordinary receptacles shall be sterilized by exposure in an autoclave at a temperature of 120° C. (15 pounds pressure) for five minutes. Where media are sterilized in large bulk, the period of heating shall be extended to 12 minutes. It is preferable, however, to sterilize media in reasonably small containers (500 to 700 c.c.).

In intermittent sterilization media shall be placed on each of three successive days in streaming steam for 30 minutes after the steam fills the sterilizer.

Reaction.⁸⁷

Phenolphthalein shall be the standard indicator used in obtaining the reaction of all media. Turmeric paper possesses similar properties, and its use is advised where phenolphthalein is not available.

Titration and adjustment of reactions shall be made as follows:

Put five c.c. of the medium to be tested into 45 c.c. distilled water. Boil briskly one minute. Add one c.c. of phenolphthalein solution (five g. of commercial salt in one liter of 50 per cent alcohol.) Titrate while hot (preferably while boiling) with $\frac{N}{40}$ caustic soda. A faint, but distinct pink color marks the true end-point. This distinct pink color may be more precisely described as a combination of 25 per cent of red (wave length approximately 658) with 75 per cent of white as shown by the disks of the color top, described under Record of Tints and Shades of Apparent Color, page 22. In practice titration is continued until the pink color of alkaline phenolphthalein matches that of the fused disks.

All reactions shall be expressed with reference to the phenolphthalein neutral point and shall be stated in percentages of normal acid or alkaline solutions required to neutralize them. Alkaline media shall be recorded with the minus (—) sign before the percentage of normal acid needed for their neutralization, and acid media with the plus (+) sign before the percentage of normal alkaline solution necessary for their neutralization.

The standard reaction of culture media shall be +1.0 per cent. If it differs from 1 per cent by more than 0.2 per cent it should be readjusted.

Wherever reactions other than the standard above given are used it shall be clearly stated in all results of bacterial work, and the reasons therefor also stated.

Storage of Media.

It is recognized by the committee that it is desirable to prepare media in large quantities in order to guard against discrepancies in composition; but, all things considered, the complications resulting from the varying amounts of heating incident to withdrawing portions from time to time and tubing it, are believed

to more than offset this advantage. Consequently, when possible, media shall be put at once into tubes and placed in cold storage.

To guard against changes due to evaporation all media not used promptly shall be stored in a moist atmosphere, preferably in an ice-box, or else the flask shall be sealed by dipping the cotton plug in paraffin.

Nutrient Broth.

Nutrient broth shall be prepared as follows: Infuse 500 g. chopped lean meat 24 hours with 1000 c.c. distilled water in refrigerator. Restore loss by evaporation. Strain infusion through cotton flannel. Add one per cent peptone. Warm on water bath, stirring until the peptone is dissolved. Heat over boiling water (or steam) bath 30 minutes. Restore loss by evaporation. Titrate. Adjust reaction to +1 per cent by adding normal hydrochloric acid or normal sodium hydrate, as required. Boil two minutes over free flame, constantly stirring. Restore loss by evaporation. Filter through absorbent cotton and cotton flannel, passing the liquid through until clear. Titrate and record final reaction. Tube, using 10 c.c. in each tube. Sterilize.

Sugar Broths.

Sugar broths shall be prepared in the same general manner as the standard nutrient broth, with the addition of one per cent of dextrose, lactose, saccharose or other sugar; or the sugar may be added to the completed nutrient broth just before sterilizing. Except in the case of dextrose broth it is important that the muscle-sugar in the meat infusion be removed by inoculating with *B. coli*.¹³⁸

The reaction of sugar broths shall be neutral to phenolphthalein.

Sterilization shall be done in streaming steam in the case of all sugar broths to prevent inversion of the sugar.

For the routine work of testing samples of water for *B. coli* especially large volumes of water are to be mixed with broths of such strength, as to make the resulting mixture one of normal strength. Liebig's Beef Extract may be substituted for beef infusion in the preparation of dextrose broth only: three grams of the beef extract for each liter of broth.

Nutrient Gelatin and Agar.

Nutrient gelatin¹⁸⁵ and agar¹⁸⁶ shall be prepared as follows:

- | | Gelatin. | Agar. |
|-----|---|---|
| 1. | | Boil 15 g. thread agar in 500 c.c. water for half an hour and make up weight to 500 g. or digest for 10 minutes in the autoclave at 110° C. Let this cool to about 60° C. |
| 2. | Infuse 500 g. lean meat 24 hours with 1000 c.c. of distilled water in refrigerator. | Infuse 500 g. lean meat 24 hours with 500 c.c. of distilled water in refrigerator. |
| 3. | Make up any loss by evaporation. | |
| 4. | Strain infusion through cotton flannel. | |
| 5. | Weigh filtered infusion. | |
| 6. | Add one per cent Witte's peptone and 10 per cent gold label sheet gelatin. | Add two per cent of Witte's peptone. |
| 7. | Warm on water bath, stirring till peptone and gelatin are dissolved and not allowing the temperature to rise above 60° C. | |
| 8. | | To 500 g. of the meat infusion add 500 c.c. of the three per cent agar, keeping the temperature below 60° C. |
| 9. | Heat over boiling water (or steam) bath for 30 minutes. | |
| 10. | Restore loss by evaporation. | |
| 11. | Titrate, after boiling one minute to expel carbonic acid. | |
| 12. | Adjust reaction to +1.0 per cent by adding normal hydrochloric acid or sodium hydrate as required. | |
| 13. | Boil two minutes over free flame, constantly stirring. | |
| 14. | Make up loss by evaporation. | |
| 15. | Filter through absorbent cotton and cotton flannel, passing the filtrate through the filter until clear. | |
| 16. | Titrate and record the final reaction. | |
| 17. | Tube, using 10 c.c. of medium in each tube. | |
| 18. | Sterilize five minutes in the autoclave at 120°, or for 30 minutes in streaming steam on three successive days. Put the gelatin at once into ice-water till solidified. | |
| 19. | Store in the ice-chest in a moist atmosphere, to prevent evaporation. | |

Lactose (or Dextrose) Litmus Agar.

Lactose or dextrose litmus agar shall be prepared in the same manner as nutrient agar, with the addition of one per cent of lactose (or dextrose) to the medium just before sterilization. The reaction shall be made neutral to phenolphthalein (see p. 106).

If the medium is to be used in tubes the sterilized azolitmin solution shall not be added until just before the final sterilization.

If the medium is to be used in Petri dishes the sterilized azolitmin solution shall not be added to the medium until it is ready to be poured into the dishes.

Milk.¹²⁷

The milk to be used as a culture medium shall be as fresh as possible, "Certified Milk" being ordinarily the best obtainable in city laboratories. It shall be placed in a refrigerator over night to allow the cream to rise and the suspended matter to settle. The skimmed milk shall be siphoned off into a flask for use. It will be found more convenient, however, to allow the milk to stand in a separating funnel. Should the milk be too acid the reaction shall be corrected to +1 per cent by the addition of normal sodium hydrate. It is then ready to be tubed and sterilized. Litmus milk shall be prepared as above, with the addition of sterile 1 per cent azolitmin. As it is impossible to make each lot of litmus milk with the same shade of color, it is recommended that a control tube be always exposed with the inoculated tubes for purposes of comparison.

Nitrate Broth.

Dissolve one gram peptone in one liter of tap water, and add two grams of nitrite-free potassium nitrate.

It is convenient to prepare a stock solution of potassium nitrate by dissolving four grams of solid nitrate in 100 c.c. of distilled water and use five c.c. of this solution in the above formula. Ten c.c. of the medium thus prepared shall be placed in a test tube and sterilized in the usual way.

Broth for Indol Test.

Standard broth may be used for the indol test if precautions are taken to remove the muscle sugar,¹²⁸ by inoculating the beef infusion with *B. coli* before making the broth.

Peptone solution,¹³⁰ (one per cent peptone in water), however, is preferred by some for use in the indol test, and is considered generally to be satisfactory. Sodium nitrite (0.01%) shall be added in all cases.¹⁴⁵

Apparatus.

Few definite requirements need be made respecting apparatus. The quality of the glass shall be such as not to be easily acted upon by the reagents used, and all glassware shall be scrupulously clean when used. When necessary it shall be sterilized by dry heat for one hour at about 150° C. A slight browning of the cotton stoppers is a good index of proper exposure.

In some operations, as, for example, the determination of the thermal death point it is necessary to use test tubes of a definite size and thickness. For this purpose the standard size culture tube shall be 15 cm. long, 1.6 cm. in diameter, and of medium weight. Tubes to be filled with gelatin for quantitative work may be those described as 6" × 5/8" "heavy."

The standard loop for making transfers shall be prepared as follows:

Bend the end of a piece of No. 27 platinum wire about 10 cm. long over a bit of No. 10 wire, and fasten the loop thus formed into a glass rod to serve as a handle. A loopful of culture shall be interpreted as meaning all the fluid that the loop can hold. That is, the fluid shall form a bi-convex body and shall not be simply a film covering the space in the loop.

The standard fermentation tube shall be a tube 1.5 cm. in diameter, bent at an acute angle, closed at one end and provided with a bulb at the other which is large enough to receive all the liquid contained in the closed portion. The length of the closed end of the tube shall be about 8 cm.

Incubation.

There shall be two standard temperatures of incubation for special work, namely, 20° C. and 37° C., the first corresponding to ordinary room temperature, the second to blood heat. The temperature of the incubators shall not be allowed to vary from these two standards by more than 1° C. in either direction.

The atmosphere of the incubator shall be kept moist,¹⁰² preferably near the point of saturation. The incubator shall be ventilated so as to insure a reasonably good circulation of air in order to prevent the accumulation in the incubator of gases which might be prejudicial to the development of the bacteria.

No definite period of incubation can be prescribed which will be suitable for all the work of species determination, but in reporting results the period used shall always be stated and form a part of the report. General statements as to the necessary periods will be found in connection with the principal tests.

*Preliminary Cultivation.*¹¹¹

It is impossible to control completely the original vitality of bacteria when ready for cultivation, because in most cases the conditions for their optimum growth are not known. Experience, however, has shown that, when bacteria are submitted to a period of preliminary cultivation or rejuvenation in nutrient broth and transfers of young cultures made from one tube to another at frequent intervals, the result is to put the bacteria into a condition where subsequent cultures give greater uniformity in their characteristics than where this procedure is not followed. The following shall be considered as the standard procedure for this preliminary cultivation, and all bacteria shall be so treated before proceeding to the detailed tests.

Procedure.—Make a transfer from an agar culture of the bacterium to be tested into a tube of nutrient broth, and incubate for 24 hours at 20° C. Transfer from this culture to a second tube of broth, and again incubate for 24 hours at 20° C. Transfer from this second culture to a third tube of broth and incubate again for 24 hours at 20° C. From this third broth culture make a gelatin plate and incubate for 48 hours at 20° C. (This is to prevent working with a possible mixed culture due to accidental contamination.) From one of the colonies on the gelatin plate transfer to a tube of slanted agar, incubate at 20° for 48 hours, and use this culture for making subsequent inoculations in the various media.

MODE OF GROWTH.

The following data represent the standard requirements for describing the mass characteristics of bacterial cultures.

Nutrient Broth.

Condition of fluid, whether clear or turbid; character of turbidity; amount of sediment, etc.; surface pellicle, color, consistency, structure; reaction and odor.

Gelatin or Agar Plates.

Surface colonies.—Form of colony; size of colony; surface elevation; topography of surface; microscopic internal structure of colony; microscopic structure of margin of colony; color; determined both by transmitted and reflected light; luster; consistency.

Deep colonies.—Form; size; microscopic structure; consistency; color; change in surrounding medium.

Gelatin or Agar Tubes.

Stab cultures.—Growth along line of puncture; surface growth (same as for plate cultures); extent of liquefaction, if any (same as for plate cultures).

Streak cultures.—Form; size; surface elevation; topography of surface; color; consistency; change in medium.

It is recommended that the descriptions of the cultures be of the briefest possible character, and that, as far as possible, botanical phraseology¹²⁷ be used.

Beyond this general recommendation no definite system of descriptive terms is hereby prescribed.

No definite period or temperature of incubation is prescribed in connection with the above determinations, but that time and temperature shall be used for each particular organism which appears to give the most characteristic cultures; and a statement of the general environmental conditions during cultivation shall form an integral part of the report. In general the period of incubation at 20° shall be four days, except in the case of gelatin tubes prepared to determine liquefaction, when the period shall be 14 days; at 37° the period shall be 48 hours.

BIOCHEMICAL REACTIONS.

The following are the standard requirements for the biochemical study of bacterial species:

Milk.

This test is designed to show the effect of enzymes and other bacterial products upon the medium.

Procedure.—Inoculate the milk and incubate at 37° C. for two days. Observe the time required to curdle, the character of the curd, i. e., whether an acid or a rennet curd, the character of the whey, the digestion* of the casein, the production of gas, and the odor.

Note.—In case no curd is formed, boil the milk for a few seconds and note whether curdling follows.

The reaction in a general way may be observed by adding one c.c. of sterile azolitmin solution to the milk before inoculation; but it must be remembered that the azolitmin itself may have an effect on the growth of the bacteria, and the practice is open to this objection. The reaction can be most accurately determined by titration, using phenolphthalein as an indicator.

Action upon Carbohydrates.

The action of bacteria upon the carbohydrates, i. e., upon dextrose, lactose, saccharose, etc., is best studied in the fermentation tube.

Procedure.—Just before using, heat the fermentation tube, containing the sugar broths, in the sterilizer, to drive off any dissolved oxygen that may be present. If on removal from the sterilizer gas bubbles are observed at the top of the closed arm remove them by decantation. When ready for use the liquid shall entirely fill the closed arm and stand at a low level in the bulb. When cool inoculate the surface of the liquid in the bulb with the culture to be tested. Incubate at 40° C. for 48 hours (or for a longer period if the gas formation is very slow). From day to day, however, observe the amount of gas, preferably with the aid of a gasometer.¹⁴⁰ At the end of the incubation remove the tube from the incubator and allow the liquid to come to room temperature. Express the amount of gas in per cent of the total volume of the closed arm. Note the degree of turbidity of the

* Shrinkage of the curd must not be mistaken for digestion.

liquid in the closed arm, determine the reaction of the liquid in the bulb by the use of litmus paper, or more accurately by titration. Fill the open bulb completely with a two per cent solution of caustic soda and cover the end of the tube with the thumb. Decant the gas into the bulb and shake in order to allow the caustic soda solution to absorb the carbonic acid. Decant the gas back into the closed arm and measure the amount of gas remaining. The difference between this and the total amount of gas represents the carbonic acid. Express the result in the per cent which the carbonic acid bears to the total gas.

Note.—In the case of those bacteria which do not grow at 37° C. the test shall be made at 20° C. and the culture allowed to grow for at least four days. The rate of gas formation shall be determined by daily measurements.

The production of gas is often erratic but it is more constant when the organism to be tested has first been submitted to a preliminary cultivation.

*Action upon Nitrates.*¹⁴¹

The object of this test is to determine whether the bacteria in question will reduce nitrites and if so whether the nitrogen will be changed to nitrite, ammonia or free nitrogen.

Procedure.—Inoculate a tube of nitrate broth and incubate for four days at 37° C. together with an uninoculated tube to serve as a blank for comparison. At the end of that period remove three c.c. of the culture to a clean test tube and add two c.c. of each of the naphthylamine solution and the sulphanilic acid solutions described under the determination of nitrates (see p. 40). The development of a red color indicates the presence of nitrites, the amount of nitrites being in proportion to the intensity of the color. This should be compared with any color that may be produced by treating the blank in a similar manner.

Remove one-half of the remaining portion of the culture to a second test tube and test for the presence of ammonia by adding a few drops of nessler's solution (see p. 35). The presence of ammonia is indicated by a yellow color or precipitate. When these tests are positive no further observations are required but when negative one of two conditions may be present—either the nitrates may have remained unchanged or they may have been reduced to free nitrogen. This can be ascertained only by deter-

mining the presence of nitrates in the remaining portion of the culture, which may be done by means of the phenolsulphonic method described in this report on p. 40. In stating the results the facts shall be recorded as to whether the nitrates were or were not reduced and, if they were reduced, whether the final product was nitrites, ammonia or free nitrogen.

Note.—The test for the reduction of nitrates is sometimes quite erratic. In case it is suspected that ammonia has been produced it must be remembered that this may have come from the organic matter of the medium and not from the nitrates. This may be ascertained by testing a broth culture for the presence of ammonia and comparing the result with that obtained with the nitrate broth. In order for the test to be of greatest value these results should be made quantitative.

*Production of Indol.*¹¹⁵

The production of indol is determined from a broth culture from which all traces of muscle sugar have been removed, or from a culture in peptone broth.

Procedure.—Inoculate the broth and incubate for four days at 37° C. At the end of that time add two drops of concentrated sulphuric acid and one c.c. of a 0.01 per cent solution of sodium nitrite and allow to stand for half an hour. The appearance of a pink color indicates the presence of indol. A blank determination should always be carried on for comparison.

Inhibition of Growth⁹⁷ by Reaction of Media.

The effect of the reaction of the medium upon the growth of the bacteria may be determined in two ways, either by growth in broth cultures of different reactions or by plate cultures of gelatin having different reactions. The latter method has the advantage of giving not only the reactions which limit growth but of showing also the optimum reaction. The various media used for this test shall be prepared in series with reactions varying by 0.5 per cent on either side of the neutral point and the series shall extend in either direction as far as may be necessary to determine the limits of growth. Should nutrient broth or gelatin not be favorable media for the development of the bacteria in question, that medium shall be used in which it finds its most favorable growth and a statement of this fact shall be included in the results.

Relation to Oxygen.

The determination of the obligative and facultative properties of bacteria in relation to oxygen shall be established under conditions of free access to the atmosphere on the one hand, and, on the other, by exclusion of the air by one of the recognized methods of anaërobic culture.^{146, 147, 148}

*Temperature Relations.*¹⁴²

The main points to be determined in studying the relations of the bacteria to temperature are :

1. The comparative activity of growth at 20° C. and 37° C.
2. The extreme limits within which development occurs.
3. The most favorable temperature for development.
4. The thermal death-point of the bacteria both in the vegetative and in the spore stage.

The collection of all these data is often impracticable but at least the relative growth at 20° C. and 37° C. and the resistance of the organism to a temperature of 80° C. shall be determined.

Procedure.—Use a 48-hour old broth culture grown when possible at 37° C., or preferably make a preliminary investigation to see if cultures grown on other media are more resistant. First heat a capacious water bath to the desired temperature and place several tubes each containing 10 c.c. of sterile nutrient broth in the water in immediate contact with the thermometer. After 15 minutes exposure to this temperature inoculate the broth with three loopfuls of the broth culture to be experimented with by simply removing the cotton plug but not removing the tube from the bath. Expose the now inoculated tubes for 15 minutes at the desired temperature and then transfer them at once to a vessel of cold water in order to cool them rapidly and prevent further action of the heat upon the bacteria. When cold, place them at a temperature favorable for development and keep them under observation for not less than seven days to ascertain if growth occurs. The most accurate results can be secured by removing the contents with a sterile pipette and making plate cultures from the heated cultures. The temperature required to destroy the species under consideration shall be determined

within 2° C.; thus, if samples are exposed to temperatures of 50°, 52°, 54°, 56°, 58°, and 60°, and it is found that development occurs after an exposure to 56° but not after exposure to 58° and 60° the thermal death point shall be given as 58°, although further study might show that it was slightly less than this.

Note.—Bacteria in a desiccated condition offer a greater resistance to high temperature than when moist, hence the thermal death point test must always be made when the bacteria are in a moist condition. In old cultures the power of resistance of many or all of the cells may be somewhat diminished. The presence or absence of spores also affects the result. This may be determined by staining or an approximate idea may be obtained by noting whether or not the culture resists a temperature of 80°. The composition and reaction of the medium in which its resistance is tested, the amount of the culture medium used for the test, and the character of the containing vessel also affect the result. The standard size of test tube recommended is described on page 110. In case any departures are made from the prescribed procedure, as, for example, by the use of Sternberg's bulbs, they shall be stated with result.

Pigment Formation.

The formation of pigment by bacteria is usually apparent in agar culture, but some organisms occur which produce pigment only on certain special media, e. g., *B. mesentericus ruber*, on potato. The color is frequently less pronounced at 37° than at 20° C., as in the case of *B. violaceus*. In determining the production of pigment, therefore, observation should be made on different media and at both 20° and 37°. The presence or absence of oxygen may also affect the result.

In addition to the use of descriptive color terms,¹¹¹ record of the tint and shade of the pigment should be made by the more exact method described on page 106.

The distinction between the pigment color of a culture and its superficial luster should be made, as well as notes upon the limitation or diffusion of the color in the culture medium.

SUPPLEMENTARY TESTS.

Many other morphological, cultural and biochemical tests are useful in special cases, but they are either so variable or, at present, so imperfectly developed that they have not been included among the standard tests.

In certain cases agglutination tests¹⁴⁹ are important.

The use of potato has not been included because of its failure to give results of diagnostic value. The use of blood serum has been omitted for the same reason, although it may be valuable with certain pathogenic forms.

At times other carbohydrates than those specified may be used with advantage.

The tests for pathogenesis¹⁵⁰ are not considered necessary in the case of ordinary water and sewage bacteria.

EXPRESSION OF RESULTS.

Having accumulated the data prescribed on the foregoing pages it remains to codify them for purposes of reference and comparison. In the past verbose descriptions of cultural and biochemical characteristics have produced great confusion in bacteriological taxonomy. In order to simplify the labor of comparing different species and their biological characters and in order to distinguish so far as possible between those characters which are of permanent differential value and those which are due to accidental variations, various forms of classification¹⁴⁸ have been suggested. Many of these have much to recommend them but in practical use most of them have been found to be too complicated. In order for any system to be of the greatest use it is necessary that the various items of the schedule be so arranged that a definite, positive or negative result shall be recorded. From the experiments made by the committee and from data accumulated from individual experiments the schedule given on p. 119 has been prepared. This tabular statement shall be considered as embodying the primary tests necessary for the classification of bacterial species. The more minute botanical descriptions of the cultures and the details of the biochemical reactions shall be considered as supplementary to it and shall be separately recorded.

In the tabular summary of primary tests a single cross (+) indicates a positive result; a double cross (#) an emphatically positive result; a zero (0) indicates a negative result; a question mark (?) indicates that the determination was uncertain or variable; a blank space indicates that no determination was made.

The subject of the classification of bacteria is being considered by another committee of the association, and the above forms of expression are recommended for use only until this committee shall have fully completed its labors. It is believed, however, that no radical changes are likely to be made in the near future.

A practical illustration of the use of the system of recording the results of species determination is shown in the appendix to this report, which gives an account of some of the coöperative work carried on by the committee along this line.

SCHEDULE OF PRIMARY TESTS FOR IDENTIFICATION OF BACTERIAL SPECIES.

		NUMBER (OR NAME) FOR IDENTIFICATION.	
Morphology	Form	{ Bacillus	+
		{ Coccus	0
		{ Spirillum	0
	Size	Diameter greater than 1 μ .	+
	Arrangement	{ United in pairs	+
		{ United in filaments	0
	Movement	Motile	+
	Structure	{ Flagella observed	+
		{ Spores observed	0
	Staining reactions	{ Stains easily with watery dyes	+
		{ Stains by Gram's Method	0
Cultural characters	Broth tube	{ Turbidity	+
		{ Sediment	+
		{ Pellicle	#
		{ Fecal odor	0
	Gelatin plate	Peculiarities of colonies	0
	Gelatin tube	{ Surface growth	+
		{ Needle growth	+
		{ Liquefaction	+
	Agar tube	{ Dull growth	-
		{ Wrinkled growth	-
		{ Chromogenesis	-
		{ Fluorescence	-
Biochemical reactions	Milk	{ Coagulation	+
		{ Degree of reaction	0
		{ Casein liquefaction	0
	Dextrose broth	Gas formation	+
	Lactose broth	Gas formation	0
	Saccharose broth	Gas formation	0
	Nitrate broth	Nitrite formation	+
	Peptone broth	Indol production	?
	Relation to oxygen	{ Aërobic	+
		{ Facultative anaërobic	+
		{ Anaërobic	0
	Relation to temperature	{ Growth at 20° C.	+
		{ Growth at 37° C.	+
	Thermal death-point below 80° C.		+

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APPENDIX.

COMPARATIVE STUDIES OF SPECIES.

Before recommending changes in the methods of determining species as set forth in the Report of the Bacteriological Committee of 1897, the Committee on Standard Methods of Water Analysis decided to make some practical tests of these methods, by ascertaining what results would be obtained by different observers working on the same species. On December 7, 1900, cultures of two species of bacteria isolated from the Brooklyn Water Supply were sent to 31 bacteriologists with the request that they determine the species, using the methods which they were accustomed to follow in their ordinary routine work. The cultures were selected almost at random from a gelatin plate and were marked A and B. Transfers were made to broth, then to gelatin plates and finally to agar slants, the latter being used for shipment.

Seventeen bacteriologists worked out these cultures in detail. Their results were presented in various forms and the labor involved in comparing them was in itself an excellent object lesson, showing the great need of uniformity of expression.

About a year later two other cultures were isolated from the Brooklyn Water Supply and sent out as before. These bacteria were marked C and D. This time, however, the request was made that each bacteriologist follow closely the procedures laid down by the Bacteriological Committee of 1897. Eleven series of results were reported in May, 1902.

The results obtained by the different observers studying these four species are shown in tabular form on the accompanying sheets, according to the method finally recommended by the committee.

It will be seen from them that, for the most part, the results of the qualitative tests were in substantial agreement but that in a number of instances there were marked discrepancies in certain of the tests. For example, in the case of nitrate reduction by one of the species, six observers reported positive results and three negative results; while in the case of indol production, four observers reported positive results and nine negative results.

COMPARATIVE RESULTS OF BACTERIOLOGICAL STUDIES OF SPECIES "A," MADE BY SEVENTEEN DIFFERENT OBSERVERS.

REFERENCE NUMBER	MORPHOLOGY					CULTURAL CHARACTERISTICS										BIOCHEMICAL REACTIONS													
	Form		Size	Arrangement	Movement	Structure		Staining Reactions		Broth Tube			Gelatin		Agar Tube				Milk			Dextrose Broth	Lactose Broth	Saccharose Broth	Nitrate Broth	Peptone Broth	Relation to Oxygen	Relation to Temperature	Growth at 37° C.
Bacillus	Coccus	Spirillum	Diameter Greater than 1 μ	United in Filaments	Motile	Flagella Observed	Spores Observed	Stains Easily with Watery Dyes	Stains by Gram's Method	Turbidity (sediment)	Pellicle	Fecal Odor	Characteristic Colonies	Plate	Tube	Wrinkled Growth	Chromogenesis	Fluorescence	Coagulation	Reaction Alkaline	Casein Liquefac- tion	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Nitrite Formation	Indol Production	Facultative Anaerobic	Growth at 37° C.
1.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Summary—	16	0	0	0	7	15	4	0	2	0	15	11	3	7	5	3	16	1	5	5	4	1	0	0	0	0	0	0	0
+ 0.....	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

+ = Positive result; 0 = Negative result.

COMPARATIVE RESULTS OF BACTERIOLOGICAL STUDIES OF SPECIES "E," MADE BY SEVENTEEN DIFFERENT OBSERVERS.

REFERENCE NUMBER	MORPHOLOGY					CULTURAL CHARACTERISTICS										BIOCHEMICAL REACTIONS																	
	Form		Size	Arrangement	Motile	Structure		Staining Reactions		Broth Tube			Gelatin		Agar Tube				Milk			Dextrose Broth	Lactose Broth	Saccharose Broth	Nitrate Broth	Peptone Broth	Relation to Oxygen	Relation to Temperature					
													Plate	Tube																			
1	Bacillus	Coccus	Spirillum	Diameter Greater than 1 μ	United in Filaments	+++	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
2	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
3	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
4	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
5	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
6	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
7	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
8	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
9	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
10	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
11	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
12	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
13	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
14	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
15	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
16	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
17	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
18	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
19	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
20	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Summary	16	0	0	0	1	16	2	0	2	0	15	4	3	1	11	12	2	1	0	1	1	12	0	14	2	0	0	0	0	0	0	0	0
+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

+ = Positive result; 0 = Negative result.

COMPARATIVE RESULTS OF BACTERIOLOGICAL STUDIES OF SPECIES "C," MADE BY ELEVEN DIFFERENT OBSERVERS.

REFERENCE NUMBER	MORPHOLOGY						CULTURAL CHARACTERISTICS										BIOCHEMICAL REACTIONS																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																					
	Form		Size	Arrangement	Motile	Movement	Structure		Staining Reactions		Broth Tube			Gelatin		Agar Tube				Milk			Dextrose Broth	Lactose Broth	Saccharose Broth	Nitrate Broth	Peptone Broth	Relation to Oxygen	Relation to Temperature	Growth at 37° C.																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																								
	Bacillus	Coccus	Spirillum	Diameter Greater than 1 μ	United in Filaments	0	1	2	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

+ = Positive result; 0 = Negative result.

COMPARATIVE RESULTS OF BACTERIOLOGICAL STUDIES OF SPECIES "D," MADE BY ELEVEN DIFFERENT OBSERVERS.

REFERENCE NUMBER	MORPHOLOGY						CULTURAL CHARACTERISTICS										BIOCHEMICAL REACTIONS																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																					
	Form		Size	Arrangement	Motile	Structure		Staining Reactions	Broth Tube			Gelatin		Agar Tube				Milk						Dextrose Broth	Lactose Broth	Saccharose Broth	Nitrate Broth	Peptone Broth	Relation to Oxygen	Relation to Temperature																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																								
						Flagella Observed	Spores Observed					Plate	Tube																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																									
1.....	Bacillus					+		Stains Easily with Watery Dyes	Turbidity (sediment)	Pellucide	Fecal Odor	Characteristic Colonies	Surface Growth	Needle Growth	Liquefaction	Dull Growth	Wrinkled Growth	Chromogenesis	Fluorescence	Coagulation	Reaction Alkaline	Casein Liquefaction	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation	Gas Formation

+ = Positive result; 0 = Negative result.

The following table shows the percentage constancy of the results for the various tests :

PERCENTAGE CONSTANCY.

	(A)		(B)		(C)		(D)	
	+	0	+	0	+	0	+	0
Morphology:								
Bacillus.....	100	0	100	0	100	0	100	0
Diameter greater than 1 μ	0	100	0	100	0	100	80	20
United in chains.....	100	0	12	88	33	67	90	10
Motile.....	100	0	100	0	20	80	100	0
Flagella observed.....	100	0	67	33	33	67	100	0
Spores observed.....	0	100	0	100	0	100	100	0
Capsules observed.....	50	50	100	0	0	100	28	72
Vacuoles observed.....					33	67	25	75
Stains easily.....								
with watery dyes.....	60	40	67	33	100	0	100	0
Stains by Gram's method.....	0	100	0	100	14	86	90	10
Cultural Features:								
Broth.....								
Turbidity (sediment).....	100	0	100	0	100	0	100	0
Pellicle.....	79	21	31	69	50	50	91	9
Fecal odor.....	37	63	33	67	60	40	27	73
Gelatin Plate.....								
Characteristic colonies.....	64	36	8	92	20	80	30	70
Gelatin Tube.....								
Surface growth.....	86	14	92	8	100	0	100	0
Needle growth.....	37	63	100	0	100	0	100	0
Liquefaction.....	100	0	13	87	0	100	100	0
Agar Tube.....								
Dull growth.....	7	93	6	94	0	100	100	0
Wrinkled growth.....	0	100	0	100	0	100	100	0
Chromogenesis.....	50	50	8	92	0	100	0	100
Fluorescence.....	9	91	7	93	20	80	0	100
Potato.....								
Visible.....	100	0	100	0	100	0	100	0
Luxuriant.....	86	14	83	17	100	0	16	84
Biochemical Reactions:								
Milk.....								
Coagulation.....	39	61	80	20	100	0	100	0
Reaction alkaline.....	33	67	0	100	0	100	15	85
Casein liquefaction.....	27	73	0	100	11	89	71	29
Blood Serum.....								
Liquefaction.....	30	70	12	88	0	100	80	20
Dextrose Broth.....								
Gas Production.....	27	73	88	12	100	0	0	100
Lactose Broth.....								
Gas production.....	12	88	25	75	100	0	0	100
Saccharose Broth.....								
Gas production.....	0	100	100	0	100	0	0	100
Nitrate Broth.....								
Nitrate reduction.....	67	33	90	10	100	0	100	0
Pepton Broth.....								
Indol production.....	31	69	15	85	33	67	29	71
Relation to oxygen.....								
Facultative anaerobic.....	100	0	100	0	100	0	70	30
Relation to temperature.....								
Growth at 20° C.....	100	0	100	0	100	0	100	0
Growth at 37° C.....	77	23	100	0	100	0	100	0
Pathogenesis.....	0	100	0	100	50	50	60	40

When the figures stand 100 in one column and 0 in another they indicate that all the results were alike; when they stand 50 and 50, they indicate that the results were equally divided. The table shows that nearly all of the tests are at times more or less erratic with certain species. Species A seemed to be more variable in its cultural features and biochemical reactions than the others. Of the tests which showed the greatest variations, the production of indol was perhaps the most marked. The average percentage of constancy

was 85 per cent, that is, on an average 85 per cent would give results in one direction and 15 per cent in the other. The average percentage constancy for the cultural and biochemical tests in the case of species A, was only 81 per cent, while in the case of species D it was 93.5 per cent.

These tabular results do not tell the whole story. Some of the details not included in the tables are given below. They emphasize the need of greater attention to details in the methods of species determination.

SPECIES "A."

Source.—Brooklyn Water Supply.

Name.—No one succeeded in identifying this form with any published description. One connected it with the bacillus liquidus group and one with the cloacae group; one mentions its resemblance to *Pseudomonas albus* (Zimmerman.) The name "*Pseudomonas nigra*" has been suggested as an appropriate one.

MORPHOLOGY.

Shape.—Short rods with rounded ends; almost oval in form.

Grouping.—On solid media it occurs singly or in pairs; in broth, short chains are numerous.

Size.—Diameter very variable; extreme lengths, 0.4 to 0.8 μ ; on agar, usual diameter is 0.6 to 0.75 μ ; in broth, 0.7 to 0.8 μ . Length is variable. Minimum, 0.7; maximum, 3.0; average, 1.25 μ .

Capsules.—Capsules were demonstrated in broth cultures by two observers; two other observers failed to observe them.

Spores.—No spores produced.

Flagella.—Four observers readily demonstrated flagella by Pitfield's method and Loeffler's method. Their descriptions substantially agree that there is a single polar flagellum, three to five times the length of the cell, thin and undulating.

Motility.—Active, darting movements.

Staining phenomena.—Stains well and uniformly with watery dyes; is decolorized by Gram's stain.

CULTURAL CHARACTERS.

Broth tube.—Broth turbid after one or two days at 20° C. After a period, variously stated from two to nine days, a thin, milky scum forms on the surface, adhering to the sides of the tube, but sinking when disturbed. There is a heavy, flocculent precipitate. Old cultures show a brownish discoloration, and have a putrefactive odor.

Agar tube.—After 48 hours at 20° C. a narrow, flat, moist, glistening, homogeneous growth of a dull white color appears. Later this becomes thicker and wider, with undulating margins and shining surface. The color becomes brownish and the medium discolored or "smoky." The medium finally becomes very dark and almost black. Some describe the color as reddish-black, others as greenish-black.

Gelatin plate.—The surface colonies at the end of 48 hours are generally described as round, five or eight mm. in diameter, crateriform, entire or having a minutely ciliate border; texture granular or grumose, greenish-white in

color. One observer mentions notable concentric rings of granular material, while another speaks of a central nucleus and a radially striate border. One speaks of a cottony sediment. Liquefaction progresses rather slowly, and the medium becomes discolored. The *submerged colonies* at the end of 48 hours are almost spherical, and about 0.5 mm. in diameter. They are coarsely granular. The borders have a loosely grumose structure, giving a jagged appearance like colonies of the amoeboid or proteus type.

Gelatin tubes.—Descriptions generally agree that after 24 hours there is a well-defined growth along the line of puncture, wider at the top, with a crateriform depression due to liquefaction of the gelatin. Liquefaction progresses slowly in the upper portions of the tube, and a heavy precipitate settles in the liquefied gelatin. After four or five days the upper half of the tube is entirely liquefied, and the funnel below is filled with a dense yellowish-brown flocculent precipitate. Some observers state that liquefaction progresses slowly, and that the growth takes the form of a narrow funnel, with little surface growth. One observer noticed that the first generation brought about a much more rapid liquefaction of the gelatin than did succeeding generations.

Potato.—Descriptions generally agree that after 48 hours the growth is scant, thin, moist, and dull white. After four days it becomes luxuriant, thick, with lobed margin. The color at first deepens to a yellowish-brown, and ultimately becomes a very dark brown, while the potato becomes black.

BIOCHEMICAL REACTIONS.

Temperature relations.—The reports of the various observers showed some disagreement. Three stated that no growths occur at 37° C., while 10 stated that growth does occur. The following quotations are taken from various reports: "The growth is more rapid but less abundant at 37° than at 20° C." "Grows well at 20°, but poorly at 37° C." "Grows well at 37°, but better at 20° C." "Grows more rapidly at 37° than at 20° C."

Milk.—The reports disagreed considerably. Eight stated that no coagulation took place, while five stated that it did occur. Three stated that acid was produced, three that alkali was produced, and two that the reaction was not changed.

Nitrate reduction.—Reports disagreed. Three stated that the nitrates were not reduced and six stated that they were reduced. Most observers stated that the nitrates were reduced to ammonia, and that no reaction was given for nitrite, while one stated that nitrates were reduced first to nitrite and then to ammonia.

Indol production.—The reports disagreed. Four stated that indol was produced, eight that it was not produced.

Action upon carbohydrates.—Most of the observers stated that no gas was produced in dextrose, lactose, or saccharose broth. Four stated, however, that gas was produced in dextrose broth and one that it was produced in lactose broth. One stated that in glucose broth 23 per cent of total gas was produced in five days at 20° C. One stated that at 20° C. a small amount of gas was produced in dextrose and lactose broth in some cultures but not in others.

Relation to oxygen.—The organism is a facultative anaërobe.

Chromogenesis and fluorescence.—Five observers reported that there is no fluorescence or chromogenesis. Four reported that the organism is chromogenic, and one that it is fluorescent.

Pathogenesis.—This was tested on a guinea pig and on a house mouse, without fatal result.

SPECIES "B."

Source.—Brooklyn Water Supply.

Name.—Two observers connect the organism with the colon type, one with the *Bacillus superficialis* type.

MORPHOLOGY.

Shape.—Occurs generally as short rods with rounded ends; sometimes oval.

Grouping.—Occurs singly and in pairs; sometimes in chains.

Size.—Diameter generally stated as 0.7 to 0.8 microns. One observer gives it as 0.5 and one as 0.3. The average length is given as 1.25 to 1.75, but lengths given vary from 0.7 to 3.0 microns.

Stain.—Stains readily with aqueous dyes. Is decolorized by Gram's method.

Capsules.—Two observers mention a capsule, and one speaks of a zoöglea formation in broth. One states that a broad uniform capsule was demonstrated by Lowitz' method.

Spores.—None produced.

Motility.—Actively motile.

Flagella.—One observer states that the flagella are peritrichial and that the average number is four. One failed to observe flagella by Pitfield's method. One mentions a single flagellum that may arise from any part of the body. (Lowitz' method.)

CULTURAL CHARACTERS.

Broth tube.—The reports agreed that broth is rendered turbid, and there is a sediment, but practically no scum.

Gelatin plate.—The reports generally agreed that in 48 hours the surface colonies are flat, irregularly round, and one to two mm. in diameter. Color is pale yellowish-white, slightly brownish in center. Later the colonies have a raised center and a slightly raised, lobed margin. The surface is somewhat granular, dull, brownish-white and translucent. General character is colon-like. Sub-surface colonies are spherical, with no special characteristics.

Gelatin tube.—The reports generally agreed that there is a slight growth along the track of the needle, which, after a time, becomes dense and beaded. The surface is somewhat similar to that on the gelatin plate. One observer mentions the formation of gas bubbles. Another states that liquefaction began on the twelfth day, and at the end of four weeks was noticed the whole length of the stab. Another states that in three weeks the gelatin was slightly liquefied, and in four weeks entirely liquefied.

Agar tube.—The reports generally agreed that there is at first a thin, moist, flat growth, grayish and translucent. Later it spreads widely as a flat

growth, with undulating margins, grayish and translucent. One report mentions a greenish fluorescence by artificial light.

Potato.—The reports generally agreed that after 48 hours the growth is thin, whitish, and shining. Later it becomes thicker, yellowish-white, moist, glistening, and luxuriant.

BIOCHEMICAL REACTIONS.

Milk.—All but two reports agreed that milk is coagulated with the production of acid.

Nitrate reduction.—All but two reports stated that nitrate is reduced. One stated that the reduction was to nitrites, another that it was to ammonia, and a third that it was reduced to both.

Indol.—All but two observers stated that no indol was produced. One report stated that "in neutral bouillon in five days there was no reaction cold, but it was evident on warming; in fourteen days there was a slight reaction cold, which became strong in warming."

Effect on Carbohydrates.

Dextrose.—All reports but two stated that gas is produced. Amounts of total gas vary from 42% to 77% in three days' incubation at 20° C. At 37° C. the amount of gas is stated as 84% to 90%. The average is about 60%. Reports of percentage of CO₂ agree quite closely. The average is 60% CO₂, but variations from 57% to 67% were noticed. There was some difference of opinion as to the production of acid. Three reports stated that the liquid in the open bulb was alkaline, two that it was acid.

Lactose.—Only two reports mentioned gas production in lactose broth. One of these states that there was 20% produced in three days at 20° C. The other states that 6% was produced.

Saccharose.—All agreed that gas is produced in saccharose broth. The amount of gas is variously stated from 50% to 100%; the average being about 70%. The per cent of CO₂ ranged from 50% to 66%, the average being about 60%. The same disagreement was recorded as to the end reaction of the medium, as was noted under dextrose.

The following is a summary of the results of gas production in the various sugar broths:

Sugar	Bacteriologists number	In-cubation days	Temperature, Degrees Cent.	Per cent total gas	Per cent CO ₂	End reaction
Dextrose.....	1	?	20	72	60	Acid
Dextrose.....	5	10	20	90	67	Alkaline
Dextrose.....	5	3	37	83	60	"
Dextrose.....	6	10	20	47	67
Dextrose.....	9	5	20	77	57	Alkaline
Dextrose.....	10	10	20	80
Dextrose.....	11	3	20	42
Lactose.....	1	?	20	10	0	Acid
Lactose.....	10	10	20	6
Saccharose.....	1	?	20	98	60	Acid
Saccharose.....	6	10	20	62	50
Saccharose.....	9	5	20	100	..	Alkaline

Temperature Relations.—All observers agreed that the organism grows well at 37° C. and 20° C.

Relation to Oxygen.—All observers agreed that the organism is a facultative anaërobe.

Chromogenesis and Fluorescence.—All but two observers gave negative replies. One mentioned a fluorescence on agar which is lost on old cultures. One mentioned a yellow growth on potato.

Odor.—Two mentioned a disagreeable odor. Five stated that there was no disagreeable odor.

Pathogenesis.—One test; negative.

SPECIES "C."

Source.—Brooklyn Water Supply.

Name.—One observer stated that the species belonged to the colon group but was not identical with the *B. coli*; another thought it to be *bacillus lactis aerogenes*.

MORPHOLOGY.

Shape.—A short, stout bacterium, with rounded ends, variable in shape and size even in the same culture. On solid media it becomes very short, almost a coccus.

Grouping.—Single, often in pairs (resembling diplococci), and rarely in chains. Upon these characteristics there was universal agreement. One observer reported that the coccoid forms were most numerous in old cultures.

Size.—The diameter, as determined from agar culture, was variously stated as follows: 1.0; 0.8; 0.9; 1.0; 0.87; 0.74; 0.7; 0.3 microns. The average was 0.85 microns. The length on agar was stated as varying from one to three, the average being approximately two. On gelatin the dimensions were reported as being somewhat less than the above, and in broth, somewhat greater.

Structure.—Spores, capsules, vacuoles, crystals, etc., were generally reported as absent. One mentioned a "clear streak surrounding the cells." Another stated the "vacuoles were apparent in watery stain from a 48-hour agar culture." One observed vacuoles.

Flagella.—One observer reported that flagella were stained by Loeffler's method. Other observers reported no flagella.

Motility.—There was universal agreement that the bacterium was non-motile in broth. One observer reported a "sluggish motility in emulsions made from agar cultures grown 24 hours at 37° C."

Staining Phenomena.—Stains readily with watery dyes. One reported that it could be stained by Gram's method, six reported the contrary.

CULTURAL CHARACTERS.

Broth Tube.—At 37° C. the medium became visibly cloudy in four hours and distinctly turbid in 10 hours. It remained turbid, not clearing in 14 days. The observers disagreed somewhat as to the formation of a pellicle.

Gelatin Plate.—There was universal agreement that the gelatin was not liquified. Sub-surface colonies were called small, sub-spherical, or lenticular,

light colored, sometimes slightly yellow, with smooth margins and fine granular texture: Surface colonies were described as nearly round, with an entire, undulating or lobed margin, with a generally flat, but contoured surface and homogeneous granular texture, color generally whitish, sometimes translucent, near the edges. The gelatin colonies resembled those of *B. coli*.

Gelatin Tube.—The reports were in substantial agreement that growth took place along the stab, spreading out slightly over the surface, the lower part of the puncture was beaded. Some observers reported gas bubbles, others did not. One stated that the growth had a doughy consistency.

Agar Tube.—There was substantial agreement that growth took place as a raised culture with smooth, glistening surface and an undulating margin. Color almost white.

Potato.—The results were not in entire agreement. It was generally stated that the growth was heavy and moist. Some described it as dull, others as glossy. Some gave the color as a light yellow, some as dirty white and some as a faint green. Some stated that the potato was discolored around the growth, others that it was not.

BIOCHEMICAL REACTIONS.

Temperature Relations.—It was generally agreed that growth took place more rapidly at 37° C. than at 20° C. The thermal death-point was variously stated as follows, 54° C., 56°, 60°, and 62°.

Milk.—The results were in substantial agreement that milk was coagulated in 12 to 15 hours at 37° C. and in four or five days at 20° C. Acid was produced. Some stated that the curd was slowly digested, others that a granular sediment was observed after three days at 37° C. Some stated that the curd was soft and was not digested.

Nitrate Reduction.—(See Table.)

Indol Production.—(See Table.)

Action upon carbohydrates.—The results were in substantial agreement that gas was produced in all three sugars. The following figures show the maximum, minimum and average percentages of total gas and the relation which the carbonic acid bears to the total gas.

	Dextrose	Lactose	Saccharose
Per cent of { Maximum.....	84	85	85
total gas { Minimum.....	54	45	60
{ Average.....	70	70	70
Per cent which { Maximum.....	67	67	67
the CO ₂ was of { Minimum.....	29	28	10
the total gas. { Average.....	52	52	47

Relation to Oxygen.—There was a general agreement that the organism is a facultative anaërobe.

Chromogenesis and Fluorescence.—(See Table.)

Pathogenesis.—(See Table.)

SPECIES "D."

Source.—Brooklyn Water Supply.

Name.—One observer reported that the organism was *B. subtilis*; one reported that it belonged to the mesentericus group.

MORPHOLOGY.

Shape.—Long rods in liquid media and short, stout rods on solid media.

Grouping.—Occurs singly and in chains of three or four individuals.

Size.—Diameter varies from 0.7 to 1.1 μ ; the average being between 0.85 and 0.95. Length varies, ordinarily from 2.0 to 4.0 μ but extremes vary from 1.5 to 10.0 μ .

Spores.—Small oval spores produced, located generally near the middle of the cell. These spores were studied somewhat carefully by Dr. Hibbert W. Hill, using the hanging block method: He reported that the free spore is elliptical and refractive, frequently showing remnants of an apparent membrane at one or both ends. The free spore enlarges rapidly in both diameters, loses its hard appearance and becomes practically indistinguishable from a very short vegetative rod. It quickly elongates and it is then possible to see at one end of it a developing rod. A faint line across the rod indicates the open end of the spore-case. The hard outline of the spore-case remains constant in size but the rod itself increases rapidly in length and diameter. After a time, the spore case slips off more or less quickly and smoothly from the enlarging rod, lying thereafter beside or behind the end of the rod but at a little distance from it, probably due to the slight jerk given as the growing tension of the cell membrane of the rod compels it to slip off. Frequently, even before the spore-case is "shed" the new rod will have divided by fission and these two rods, now separate at their proximal ends, one bowing a little and slipping past the other on the side opposite the concavity of the bow. (This "slipping by" occurs in both Species C and Species D.) The rods tend ultimately to become parallel but fission sometimes proceeds so rapidly that chains result, the component rods of which sometimes buckle out of line and then pass each other as above described. The rods in D often contain very numerous granules, which may be precursors of spores.

Capsules.—No capsules were observed by staining, but in some cases the reports stated that the bacteria were surrounded by clear zones.

Flagella.—Flagella were demonstrated by two observers. In both cases they were reported as peritrichic.

Motility.—The organism moves with a more or less direct undulating or wobbling motion.

Staining Phenomena.—The organism stains well and uniformly with watery dyes and also by Gram's stain.

CULTURAL CHARACTERS.

Broth Tube.—Broth at first turbid, afterwards clear. Heavy light colored precipitate. Pellicle is formed which breaks easily and sinks to the bottom.

Agar Tube.—The reports generally agreed that the growth on agar was luxuriant, spreading, slightly raised, dull, wrinkled, opaque, white and slightly opalescent.

Gelatin Plate.—The descriptions generally agreed that the gelatin is liquefied, that the surface colonies are round and that cottony or slightly brownish masses float around in the liquid medium. Margin, rather smooth.

Gelatin Tube.—The descriptions generally agreed that liquefaction takes place first near the surface and ultimately proceeds down the tube in a stratiform manner.

Potato.—The growth on potato is a wrinkled dull dirty white mass.

BIOCHEMICAL REACTIONS.

Milk.—The reports generally agree that milk was promptly coagulated at 37° C. Most observers stated that subsequently the casein becomes digested.

Nitrate Reduction.—(See Table.)

Indol Production.—(See Table.)

Action upon carbohydrates.—The reports agreed that no fermentation of the sugars takes place.

Relation to Oxygen.—The organism was generally reported as a facultative anaerobic form, although a few observers described it as aerobic.

Chromogenesis and Fluorescence.—(See Table.)

Pathogenesis.—(See Table.)

CONCERNING TESTS FOR B. COLI COMMUNIS IN WATER.

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THE writers of this paper were members of a joint commission* which, on behalf of the city and of the water company, reported last autumn at Indianapolis upon the character of the public water supply. The investigation embodied a comprehensive study of the physical features of the water works system, an inquiry into the prevalence of typhoid fever and all the leading factors relating thereto, and a bacteriological examination of the city water supply. Daily tests of numerous samples of city water were made for a period of more than 30 days.

In the course of these investigations there were encountered a number of features which it is believed are worth recording briefly, although they are not essentially different from those experienced in some other instances. The points mentioned refer particularly to tests for B. coli, and these will be described following a brief outline of the works from which the public supply was at that time derived.

The major portion of the public water supply was derived from a series of deep wells, driven in the rock some 300 feet or so. The well water was delivered by a Pohle airlift system into an open reservoir, the sides and bottom of which were not water tight. Connected to this reservoir, which was quite near White River, was a gallery about 1,000 feet long, built diagonally along the shore and below the ordinary water level in the river. When the water was lower in the gallery than it was in the river, some river water passed into the gallery and reservoir after partial filtration through the intervening layers of porous sand and gravel.

*See Report of Oct. 28, 1904, on the Water Supply and Sanitary Conditions of Indianapolis, by Messrs. G. W. Fuller, C. E. Ferguson and B. J. T. Jeup.

When the water in the gallery and reservoir became higher than it was in the river, there was obviously a flow in the opposite direction. During these investigations a portion of the new sand filter plant was put in service, and filtered water from Fall Creek formed a small part of the public supply.

The well water contains considerable iron, which very slowly deposits, forming within the pipes a sediment which becomes stirred up at times of very high velocities within the piping, such as during fires, etc. This sediment caused some irregularities in the bacterial contents of the water. To perhaps an unusual degree the water within the distributing system contained bacteria which were in a somewhat degenerate form. In part this seemed due to species which formed the 5 or 10 bacteria per c.c. present in the well water, coming in part, no doubt, from the air used in lifting the water from the wells. In part they seemed to be associated with the bacteria in the porous soil adjoining the reservoir and gallery, through which the water moved in opposite directions from time to time. Bacterial growths also took place to some extent in the gallery.

The number of bacteria per c.c. in water from different sources was found to average, for a period of 30 days, as follows:

	Bacteria per c.c.
White River - - - - -	350
Filter Gallery - - - - -	375
Gallery Reservoir - - - - -	120
Driven Wells - - - - -	4
City Tap Water - - - - -	100

I.

The first point to which it is desired to call attention is the unreliability of the so-called presumptive colon tests under the existing local conditions. *B. coli* was repeatedly found to be present in fermentation tubes in which the amount of gas was less than 20 per cent or even 10 per cent after a period of incubation of 48 hours at 37° C.

The procedures followed in this work were substantially those recommended by the Bacteriological Committee of the American Public Health Association. Glucose solutions, however, were

prepared with meat extract rather than with meat infusion. The reaction of the media during the early part of the work was neutral to phenolphthalein, but later the reaction was made + 0.5 per cent. There was not much difference, however, in the two reactions used, although some special tests indicated that for this hard water (about 300 parts per million total hardness) the neutral reaction was not quite so satisfactory as in many places where softer waters are used.

In all 410 samples of water were examined for the presence of colon bacilli, in most cases one c.c. and five c.c. of each sample being tested. Colon bacilli were detected in 43 samples; 30 of them were in one c.c. portions, and the remainder in five c.c. portions.

In all cases there was a pure culture made from the fermentation tube and subcultures made on gelatin tubes, agar slants, dextrose broth (fermentation tube), milk, pepton solution (indol) and nitrate solution. Data from all these tests as well as the morphology were obtained before deciding on the identity of a species.

In 18 out of the 43 samples containing *B. coli* the volume of gas in the original fermentation tube was less than 20 per cent at the end of 48 hours, and in 11 instances it was less than 10 per cent. These figures indicate a serious error into which the investigation might have fallen if the records had been based on presumptive tests.

II.

During the latter half of the investigation transfers were made on the morning of the second day into fresh fermentation tubes in the instance of each sample which had shown any sign of gas after having been incubated for a period of 15 to 18 hours at 37° C.

The second set of fermentation tubes was incubated for 48 hours at 37° C. The comparison of results so obtained with those from the tubes in which the samples of water were originally placed showed a variety of differences. In some cases the percentage of gas increased from below to within the conventional limits for presumptive tests, that is, 25 to 70 per cent; in others the gas increased from within to above the limits stated;

in others there were reversals in each of the two last mentioned sets of conditions; and finally there were occasional changes from below 25 to above 70 per cent, and, vice versa. In repeated instances, of course, the percentage of gas remained approximately the same.

Observations from day to day of the irregularities above described deeply impressed the writers with the uncertainties associated with the use of mixed cultures in obtaining such biochemical reactions. The effects of overgrowth, antagonism, symbiosis and other features which have been written about from time to time for several years are given an opportunity to exercise their influence in a manner which the writers believe is prejudicial, under many cases, at least, to obtaining reliable data as to the distribution and prevalence of colon bacilli in waters.

If two or more gas-producing organisms are present in the sample, there are no reliable means of determining in advance which will have gained the upper hand of the other at any particular time, although, as is frequently the practice, it is advisable to remove for examination a portion of a tube which shows signs of gas in 15 to 18 hours, rather than to allow from 24 to 48 hours to elapse before transferring a portion for examination.

When consideration is given to the effect in a fermentation tube of bacterial species other than those which produce gas, of relative states of vitality, etc., some comprehension is obtained of the hopelessness of arriving regularly at reliable data by these means.

In making the above statements the writers are aware that the degenerate forms of *B. coli* present in many waters behave quite differently in some respects in the laboratory from colon forms taken directly from sewage or from the bodies of men or animals.

III.

Quite frequently it has been the experience that positive tests are obtained for colon bacilli in one c.c. volumes when negative results are recorded for larger ones. In the light of the experiences above described, and of our growing knowledge regarding the effect of overgrowths, antagonism, etc., it appears to the

writers that it is necessary to recast present views as to colon tests, making more numerous examinations of smaller volumes rather than to attempt to get anything reliable from the examination of larger unit volumes. This refers, of course, essentially to the use of the fermentation tube for the preliminary treatment of the water sample. Where feasible, the direct plating of the sample on agar seems preferable.

IV.

During the progress of this work the writers were unaware of the marked step in advance resulting from the use of porous earthenware covers¹ for the Petri dishes. Spreading colonies to rather an unusual degree were encountered in the Indianapolis work, thus making it a laborious task to obtain in purity cultures of the leading species producing gas in a fermentation tube. Under these circumstances great care is required, otherwise the spreading colonies will mask the presence of *B. coli*.

It appears that with the large elimination of spreading cultures by the absorption of moisture with earthen covers, there is an important field of investigation opened for improvement in the technique of coli determination. It would seem that more and more attention should be given to the direct plating of samples on agar, rather than to the use of the preliminary fermentation tube with the complications which it presents as above outlined.

It especially suggests the use of technique by which there may be isolated species producing gas, thus confining the work of searching for *B. coli* on agar plates to those gas-producing forms rather than to those which produce acid. With most workers the litmus agar plates now and then show well the gas-producing colonies, but the present technique does not seem to keep this feature uniformly under control. The use of other sugars than lactose in agar, and the testing before use of each lot of agar with a stock culture of *B. coli* to insure favorable conditions and technique for gas production, are measures which seem to promise better quantitative results.

¹ HILL, *Jour. of Med. Research*, 1904, 8, p. 93.

CHARACTERISTICS OF COLON BACILLI AND THE VALUE OF THE PRESUMPTIVE TEST.

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(From the Research Laboratory.)

IN November, 1903, an investigation was begun under the direction of Dr. William H. Park, in order to study anew what has already been the subject of considerable research work, namely, the colon-like and sewage streptococcus types of normal, and incidentally of diarrheal stools; how far the typical *B. coli* organism can be safely used as an index of pollution; and whether the presumptive test, as still applied in some important laboratories, can be relied upon as giving a fair basis for a reasonably correct judgment of waters used as sources of drinking water supply.

In the first experiments nine specimens of normal human stools from as many sources were examined. In each case a flask containing 100 c.c. of sterile tap water was inoculated with a large loopful of feces, and plates made with .1 and 1 c.c. of water from each flask directly and at intervals varying from two days to a week and extending over a period of two and a half to three months.

It will be seen from Table 1 that seven, or 77 per cent of the first series of plates made from one c.c. of the diluted feces in the nine inoculated flasks show evidence of *B. coli*, and of the six plated at the end of six weeks, five still contain abundant colon bacilli. Of those plated at the end of 10 weeks, more than 50 per cent show *B. coli* to be still abundant.

From two of the nine original flasks no colon-like colonies, in fact no gas producers whatever, were obtained in the quantity used at the first plating; one gave them subsequently, while the other gave none, at any time during the 10 weeks that the flasks were under observation. It is interesting to note that organisms of the sewage streptococcus type were found to be present in

TABLE 1.

PERCENTAGE OF COLONIES OF COLON-LIKE AND SEWAGE STREPTOCOCCUS TYPES DEVELOPING IN NUTRIENT AGAR AT 37° C. FROM SUCCESSIVE PLATINGS FROM FLASKS INOCULATED WITH NORMAL STOOLS.

	FLASK 1			FLASK 2		
	Time	Per Cent of Colon-like Colonies	Per Cent of Sewage Streptococcus Type	Time	Per Cent of Colon-like Colonies	Per Cent of Sewage Streptococcus Type
First plating.....	0	84	4	0	6	6
Second plating.....	3 days	90	0	3 days	100	0
Third plating.....	8	78	0	8 "	98	0
Fourth plating.....	10 "	100	0	10 "	74	0
Fifth plating.....	16	100	0	16 "	26	0
Sixth plating.....	—	—	—	—	—	—
Seventh plating.....	—	—	—	—	—	—
	FLASK 3			FLASK 4		
	Time	Per Cent of Colon-like Colonies	Per Cent of Sewage Streptococcus Type	Time	Per Cent of Colon-like Colonies	Per Cent of Sewage Streptococcus Type
First plating.....	0	63	0	0	71	7
Second plating.....	2 days	0	0	2 days	63	18
Third plating.....	5 "	73	0	9 "	10	0
Fourth plating.....	15 "	89	0	16 "	0	0
Fifth plating.....	3 weeks	53	0	6 weeks	25	0
Sixth plating.....	7 "	60	0	10 "	5	0
Seventh plating.....	11 "	82	0	—	—	—
	FLASK 5			FLASK 6		
	Time	Per Cent of Colon-like Colonies	Per Cent of Sewage Streptococcus Type	Time	Per Cent of Colon-like Colonies	Per Cent of Sewage Streptococcus Type
First plating.....	0	100	0	0	0	12
Second plating.....	8 days	0	0	7 days	0	10
Third plating.....	15 "	0	0	14 "	0	92
Fourth plating.....	6 weeks	0	0	6 weeks	0	93
Fifth plating.....	10 "	0	0	10 "	0	75
Sixth plating.....	—	—	—	—	—	—
Seventh plating.....	—	—	—	—	—	—
	FLASK 7			FLASK 8		
	Time	Per Cent of Colon-like Colonies	Per Cent of Sewage Streptococcus Type	Time	Per Cent of Colon-like Colonies	Per Cent of Sewage Streptococcus Type
First plating.....	0 days	71	19	0	0	4
Second plating.....	7 "	50	0	5 days	26	4
Third plating.....	14 "	30	5	12 "	35	0
Fourth plating.....	6 weeks	93	0	6 weeks	80	20
Fifth plating.....	10 "	85	0	10 "	80	5
Sixth plating.....	—	—	—	—	—	—
Seventh plating.....	—	—	—	—	—	—
	FLASK 9			No streptococcus actually isolated in this case.		
	Time	Per Cent of Colon-like Colonies	Per Cent of Sewage Streptococcus Type			
First plating.....	0	36	32			
Second plating.....	5 days	0	0			
Third plating.....	12 "	4	0			
Fourth plating.....	6 weeks	81	0			
Fifth plating.....	10 "	0	10			
Sixth plating.....	—	—	—			
Seventh plating.....	—	—	—			

every series of plates made from the flask containing no colon bacilli. Although this experiment is made with only nine samples of normal stools, the statement that *B. coli* will be abundantly present in waters subjected to multiple human pollution will be but a fair deduction from the table. It is also evident that in those cases where pollution from but one person occurred, and where *B. coli* was not found in any series of plates, that in the majority of cases organisms of the sewage streptococcus type would have given at least an indication of pollution.

Agglutination Experiments.—A group of 14 colon-like organisms, taken at random from the feces of the nine cases under discussion, and of course all of them unquestionably intestinal bacteria, were examined for agglutination reactions, and attempts were made to obtain with them an agglutinating serum for *B. coli* which should be diagnostic.

In carrying out this part of the investigation a large number of rabbits were inoculated by us with separate strains of coli, and several with mixed strains of coli cultures. The rabbits were bled and their serum tested for agglutinating reactions with the 14 cultures just mentioned. No original rabbit serum reacted strongly with any culture in a higher dilution than 1:50.

By successive inoculations of a mixture of two or three coli strains for each rabbit, it was found by testing the sera that specific agglutinins for just those strains which had been used could be raised.

Indeed, for the majority of the cultures, other than those injected, no appreciable amount of non-specific or group agglutinins developed. Only a few cultures of those not injected agglutinated slightly in the sera in 1:20 dilutions, and in most cases these cultures were derived from the same person as one of the cultures used in immunizing the animals. This result does not harmonize with the belief expressed by some that even though cultures of *B. coli*, isolated from different persons, reacted to different specific agglutinins, yet among the total specific agglutinins acting on each *B. coli* there would be one or more acting on all or at least on a large number. In our experience no more common agglutinins are found among many different

cultures of *B. coli* than are common to a strain of *B. coli* and cultures of the mannite fermenting para-dysentery bacilli.

The results of our work will be found in Table 2.

TABLE 2.
AGGLUTINATION REACTIONS WITH GROUP OF 14 COLI STRAINS FROM NORMAL STOOLS.

RABBIT 1 (Inoculated with Coli Strains 1, 7 and 12)					RABBIT 2 (Inoculated with Coli Strains 3, 4 and 10)				
	1:100	1:200	1:500	1:1000		1:100	1:200	1:500	1:1000
1	+1	+	±		3		+1	±	1
7	+1	+1	±		4	++	+	±	1
12	+	+	±	1	10	++	++	+1	+
With eleven other Coli strains a negative or only slight reaction was obtained at 1:20. ++ signifies a complete reaction. + signifies a good reaction. 1 signifies a slight reaction.					With strains 1 and 2 a fair reaction was obtained at 1:50; with nine other Coli strains a negative or only slight reaction was obtained at 1:20.				
RABBIT 3 (Inoculated with Coli Strains 2, 8 and 13)					RABBIT 4 (Inoculated with Coli Strains 5, 6 and 14)				
	1:100	1:200	1:500	1:1000		1:100	1:200	1:500	1:1000
2	++	++	++	±	5	+1	+1		
8	+1	+	+		6	±	±	±	±
13	+	±	±		14	±	1		
With strains 1 and 7 a fair reaction was obtained at 1:50; with 12 a fair reaction at 1:20; with eight others a negative or only slight reaction was obtained at 1:20.					With strain 1 a fair reaction was obtained at 1:50. With eleven others a negative or only slight reaction was obtained at 1:20.				
RABBIT 5 (Inoculated with Strains 9 and 11)									
	1:100	1:200	1:500	1:1000					
9	+	±							
11	±	1							
With strain 10 a fair reaction was obtained at 1:50. With eleven other cultures only a negative or slight reaction was obtained at 1:20.									

Other rabbits were inoculated with single strains, but in no case were agglutinins developed which were specific for members of the coli group. The sera obtained were only specific for the single strain used, and possibly specific to a smaller extent to another organism from the same specimen of stools.

Horse sera obtained by the inoculation of dysentery cultures was used to test these fourteen organisms under discussion as well as 10 to 20 other coli strains.

Horse serum 221 gave the following results with the 14 cultures under discussion:

	1:100	1:200	1:500	1:1000
1.....	+1	+	1	—
2.....	1	—	—	—
7.....	+	±	1	—
10.....	+	±	1	—
12.....	+	+	1	—

With seven other strains a negative or only slight agglutinin reaction was obtained at 1:20, and with the remaining two a fair and slight reaction at 1:50.

With 23 additional coli cultures from abnormal stools four gave no reaction at 1:20, six showed a tendency to agglutinate at 1:50, eight still showed a tendency at 1:100, four at 1:200; one, Colon Y, still gave a good reaction at 1:500, and showed a tendency at 1:1000. This one organism, Colon Y, has been of especial interest in our laboratory since it was isolated from diarrheal stools from several cases. It conforms absolutely to the rigid cultural tests for *B. coli*, and behaves in its agglutination reactions very much like the Flexner Manila culture of paradyseutery. Colon X rabbit serum, while agglutinating its own organism in a dilution of 1:1000, agglutinated strain 2 to 1:100 completely, gave a good reaction with strain 7 at 1:500, no reaction with strains 3 and 4, and only a fair reaction at 1:50 with strains 10:12.

Further attempts to obtain a serum which should be specific for the colon group were then abandoned. We are inclined to the belief that just as there are innumerable coli strains, so there may be as many specific agglutinins, and any attempt to classify coli by agglutination reactions would be correspondingly futile. It would be equally incorrect to refuse to include in the colon group any bacillus because it failed to be agglutinated by a serum which had been obtained from an animal which had received numerous strains of *B. coli*.

BIOCHEMICAL CHARACTERISTICS.

In addition to the 14 coli cultures mentioned in connection with the agglutination experiments, 34 other strains of coli were studied, the entire number, 48, being obtained from 34 different specimens of stools from as many persons. These 48 strains are, therefore, of intestinal origin and are all members of the colon group even if a very few do not conform strictly to every test for *B. coli*. All produce gas in dextrose broth, all but two are acid producers, and none liquefy gelatin in 10 to 14 days. All but four produce indol in three days, all produce nitrites in nitrate solution, all but five produce gas in neutral red lactose broth. Twelve out of 30 that were tested on saccharose broth gave no gas, or only a bubble, 18 giving gas which varied from 1 to 50 per cent in amount. The gas produced in dextrose broth by these 48 cultures varies from 15 to 90 per cent of the closed arm.

2 gave 15 %	7 gave 40 %	2 gave 60 %
2 " 20	2 " 45	1 " 65
6 " 25	10 " 50	2 " 75
4 " 30	1 " 55	1 " 90
8 " 35		

Of the total gas collected in the closed arm of the fermentation tube Co_2 formed from a trace to 65 per cent. H_2 was constantly present. The tests of these bacilli, which undoubtedly belong to the human colon group, indicate that different strains produce a widely different amount of gas and, to some extent, of proportion of Co_2 to H_2 . Fifty colonies having the characteristics of colon bacilli were fished from a lactose litmus agar plate which had been inoculated directly with normal feces from a single case. These 50 cultures were then inoculated into saccharose, lactose, dextrose, and mannite with the following results:

All 50 cultures fermented mannite with the production of acid and of visible gas, coagulated milk within three days, and produced indol within the same time; none of them changed gelatin within a period of 10 days. All changed neutral red to yellow red in lactose broth and caused a gas production of between 20 and 30 per cent of the height of the closed arm of the fermentation tube. The amount of gas in dextrose varied between 20 and

60 per cent, of which *Co.*, composed 20 to 60 per cent. The following division of gas amount was noted: 20 per cent, 20; 25 per cent, 12; 30 per cent, 7; 35 per cent, 4; 40 per cent, 4; 50 per cent, 2; and 60 per cent, 1.

The chief difference was noted in saccharose broth. In this sugar 23 produced visible gas and 27 produced none. Of the 23 producing gas, only 18 produced appreciable acid. Of the 27 producing no gas, 18 produced acid. The gas amount in the acid producing cultures varied from 1 to 10 per cent. In the cultures producing an excess of alkali, 1 to 40 per cent. Of the cultures producing the greatest amount of gas in dextrose and lactose, two produced no visible gas in saccharose.

Of the 98 *B. coli* cultures studied, only nine would be ruled out of the strict *B. coli* type, and of these five conform to every test but that of fermenting lactose, and four to every test except that of indol production. Seven of the nine were obtained from diarrheal stools. In fact, these slightly irregular *coli* types seem to be more frequently encountered in abnormal than in normal stools.

Passing now from the study of colon types as found in the human intestines to the examination of drinking waters suspected of pollution, we see, as has already been shown, that a search for *B. coli* and the sewage streptococcus would hardly fail to reveal pollution. After some preliminary work with the presumptive test for *B. coli* in dextrose broth, which will be discussed again later, we were led to the conclusion, that while the correct percentage of total gas and a proper gas ratio might really indicate the presence of *B. coli*, yet the reverse proposition, that an incorrect percentage of total gas and an improper gas ratio precludes the presence of *B. coli*, cannot be held.

In the examinations of Croton tap water we used the Lawrence method for *B. coli* identification as developed and used by Mr. Gage, and in every case where *B. coli* is spoken of as being found the cultures were fully identified at the laboratory.

The value of the so-called presumptive test for *B. coli*, depending, as it does, on the amount of gas formed in fermentation tubes from dextrose broth and the proportion of carbon dioxide

and hydrogen in the gas formed, would unquestionably be great, if it could be proved to be accurate as well as rapid. Gage (Massachusetts State Board of Health, 1902), however, finds that certain waters of known purity would be condemned by the presumptive test, while in the examination of the shellfish very erroneous results would be obtained, if the presumptive test alone were used.

Prescott and Winslow in their book, "Elements of Water Bacteriology," speak of the value as a presumptive test of the gas formation in dextrose broth. They admit that the distinction between positive and negative is not absolute, but they consider Whipple's results, obtained by examining a number of surface water supplies, as being very striking and in general sound.

In our experience we have found that our own municipal supply, with its main reservoirs and lakes, guarded carefully, and many small streams in the region of Mt. Kisco and Katonah, left comparatively unprotected and open to definite pollution, is never free from coli. So far as we have tested, it is constantly present in amounts of 10 c.c., and frequently in 1 c.c. The filling of the new reservoir is expected to take place in April. This will give longer storage to the water and thus improve its quality. These cultures were isolated by us by the method used by Mr. Gage at the Lawrence Experiment Station. The method, as modified by us, consists in inoculating dextrose broth with varying amounts of water under examination, and plating out on lactose litmus agar from any tubes showing evidence of fermentation after 16:18 hours' growth at 37° C. Thus in 48 hours lactose litmus agar plates will be obtained which, to the trained observer, will give a better basis for a preliminary judgment of the water being tested than the preliminary fermentation test alone, and colonies picked from these lactose litmus agar plates can be transferred to agar and carried through the confirmatory tests for *B. coli* as a matter of routine procedure. We present a table (Table 3), giving in parallel columns the results of *B. coli* cultures isolated from Croton water and confirmed by the Lawrence method, in column 1, and results of presumptive tests given in column 2. The *B. coli* cultures were all isolated from samples taken by

us at the tap in the Research Laboratory, foot of East 16th Street, and a few from the tap at City Hall park. The presumptive tests, on the other hand, were made on samples taken from the 135th Street Gate House. The table has the additional value, even though the results are few in number, of giving under the heading of the presumptive tests the published reports* of the Water Supply Department, and their opinion of the water was based on Mr. Whipple's provisional interpretation of presumptive tests given out in 1903, yet, while the presumptive tests are almost uniformly negative, *B. coli* has been constantly isolated by us in quantities varying from 1 c.c. to 10 c.c. The Water Supply Department had further stated that considerable purification takes place between 135th Street Gate House and the City Hall. At 16th Street, which is midway between the two stations, we should expect to get water that had at least half the purification that the City Hall water would show and therefore purer than at 195th Street.

TABLE 3.

Date 1904	Coli Cultures Isolated at Research Lab. from Croton Water	Quantity and Sources	Water Supply Reports Based on Presumptive Tests		
			c.c. 0.1	c.c. 1.0	c.c. 10.0
Sept. 6.....	Croton 1	1 c.c. E. 16th St.	0.1	1.0	10.0
	" 2		0	0	0
	" 3				
	" 4				
Sept. 13.....	" 8	1 c.c. E. 16th St.	0	0	0
Sept. 20.....	" 9, 10, 11	1 c.c. E. 16th St.	0	0	0
Sept. 28.....	" 13, 14	10 c.c. E. 16th St.	0	0	0
Oct. 4.....	" 15				
	" 18 (City Hall)	10 c.c. E. 16th St.	0	0	0
Oct. 11.....	" 19, 20, 21	1 c.c. E. 16th St.	0	0	0
	" 22, 23	10 c.c. City Hall	0	0	0
Oct. 18.....	" 24, 25	1 c.c. E. 16th St.	0	+	+
	" 26, 27	1 c.c. City Hall			
Nov. 23.....	" 28, 29	1 c.c. E. 16th St.	0	0	0
Nov. 25.....	" 31, 32, 33	1 c.c. "	0	0	0
Nov. 26.....	" 34, 35	100 c.c. "	0	0	0
		(10 c.c. not tested)			
Nov. 28.....	" 36, 37, 38, 39	10 c.c. E. 16th St.	0	0	0
Nov. 29.....	" 40, 41	10 c.c. "	0	0	0
Nov. 30.....	" 42	1 c.c. "	0	0	+
	" 43, 44	10 c.c. "	0	0	+
Dec. 1.....	" 45, 46	40 c.c. "	0	+	0
Dec. 2.....	" 47, 48	10 c.c. "	0	0	0
Dec. 5.....	" 49, 50, 51, 52	10 c.c. "	0	0	0
Dec. 6.....	" 53, 54	10 c.c. "	0	0	0
Dec. 7.....	" 57, 58, 59, 60	10 c.c. "	0	0	+
Dec. 8.....	" 61, 62	10 c.c. "			
	" 63	10 c.c. "	0	0	+
Dec. 9.....	" 64, 65, 66	10 c.c. "	0	0	0

* We are indebted to Dr. Jackson, Director of the laboratory, for these reports.

We have repeatedly found that waters giving a positive result with presumptive test in .1 c.c. have given a negative result with 1 c.c. and with 10 c.c., the growth of a considerable number of varieties of bacteria interfering with the action of the colon bacilli.

A presumptive test which frequently fails completely to reveal the presence of *B. coli* where it is a comparatively simple matter to find it by another method is certainly of questionable value, and should be subjected to rigid investigation before basing any very definite opinions on the quality of the water supply under examination.

THE CHEMICAL PHASES OF A WATER SOFTENING PROBLEM.

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THE application of methods of analysis, and their interpretation with reference to problems of water softening, depend chiefly upon the relative amounts of calcium and magnesium which the water contains. Since the composition of water is in a large measure dependent upon geological conditions, it would not appear to be possible to apply general methods of analysis, or to expect that any one method of interpreting analyses would be universally applicable. In a study of the character of a portion of the water supply of the city of Columbus, Ohio, several analytical features were developed, which illustrate the limitations of general methods of analysis for waters containing considerable quantities of magnesium.

As water softening is probably of growing importance in connection with the hygienic purification of hard waters, it seemed to the writer that an outline of the chemical phases of one of these water-softening problems might be of general interest. The suggested method of stating the results of the analysis of water for softening purposes is proposed with the hope that it may add strength to the plea for the adoption of a procedure for expressing the analytical data necessary to calculate the chemical treatment of a water, which shall be merely a statement of facts analytically determined.

THE STATUS OF THE MINERAL ANALYSIS OF WATER.

The analysis of water for softening purposes essentially differs from a sanitary analysis, in that it is chiefly concerned with the dissolved inorganic substances which constitute hardness. The relative amounts of calcium and magnesium forming the alkalinity and incrustants are the principal features of an analysis of this character. Computations of the chemicals required to soften a hard water have generally been based upon the assembled

results of a gravimetric analysis of the dissolved mineral constituents. While there can be no question as to the reliability of these data in so far as they relate to the total quantities of calcium, magnesium, and the acid radicals present, respectively, as soon as it becomes a question of deducing from these data the amounts of the specific salts of these bases conceived to exist in solution in the water, such a variety of views exists as to the procedure to be followed that different observers, working upon the same water, rarely report concordant results.

The question of the interpretation of a mineral analysis of water has been the subject under discussion in the more recent papers on water softening, the conclusions of the authors suggesting greater simplicity in expressing results, urging that only the facts of the analysis be reported, and demonstrating that the salt combinations are unnecessary for calculating a softening treatment. Thus McGill,¹ in a paper on "Water Treatment," discusses the vexed question of the mineral analysis, and urges that results be reported in terms of the oxides of the bases, and in terms of the acid radicals present, showing also that these data, supplemented by the determination of the alkalinity and the incrustants, respectively, are sufficient for making calculations of the chemicals required to soften a given water.

Handy,² in an article on "Water Softening," suggests the wisdom, in certain cases, of expressing the results as uncombined mineral constituents, thus departing from the older method of reporting the bases and the acid radicals in combination, since the complexity of many waters renders data thus expressed practically matters of conjecture.

A study of the composition of a portion of the water supply of the city of Columbus, Ohio, has shown the applicability to this water, under the conditions existing during the investigation, of a form of expressing the results of its analysis, especially relative to softening, in which the bases alone are reported. As an illustration of the facility with which the treatment of this water may be calculated from an analysis thus expressed, the necessary data

¹ *Bull. No. 55 Amer. Ry. Eng. and Maintenance of Way Association.*

² *Proc. Eng. Soc. Western Penna., December, 1903.*

and the methods of analysis employed in their determination are now presented in detail.

DATA NECESSARY FOR SOFTENING CALCULATIONS AND METHODS
USED FOR THEIR DETERMINATION.

To calculate the amounts of chemicals necessary for the treatment of a magnesium water, such as the one under study, the following data are required:

- The free and the half-bound carbonic acid.
- The alkalinity.
- The incrustants.
- The total magnesium.
- The total calcium.
- The incrusting calcium.

The free and the half-bound carbonic acid and the alkalinity were determined by the usual methods; the incrustants by Soda Reagent; the total magnesium by a modification of Pfeifer's method, which consists in precipitating the magnesium with an excess of standard lime water, and determining the excess by titration with standard sulphuric acid; the total calcium by the usual gravimetric method, and also by calculation from the total hardness; the incrusting calcium by a gravimetric determination of the calcium not removed by boiling, correcting for the calcium present due to dissolved normal carbonate.

The determination of total magnesium.—Into a porcelain evaporating dish measure 100 c.c. of the water, and add that amount of $\frac{N}{10}$ sulphuric acid required to neutralize the alkalinity. Boil to a volume of about 50 c.c. to expel the carbonic acid, and transfer the solution to a 200 c.c. flask of Jena glass, graduated at 205 c.c. and provided with a ground glass stopper. Introduce by means of a pipette 25 c.c. of clear saturated lime water. (The amount of calcium oxide present must be 50 per cent in excess of the amount required to precipitate the magnesium.) Quickly make up the volume to 205 c.c. with boiling distilled water, stopper the flask, and mix thoroughly. Place the flask on the water bath for 15 minutes, cool, and allow the precipitate to settle completely. Pipette off 100 c.c. of the clear solution into a 100 c.c. nessler tube containing slightly less $\frac{N}{50}$ sulphuric acid than

will neutralize the excess of calcium oxide, titrate with $\frac{N}{80}$ sulphuric acid, with phenolphthalein as the indicator, using the glass stirrer and observing the same precautions as in the Sehler method for the determination of free carbonic acid. Owing to the effect of temperature upon the solubility of calcium oxide, it has been found to be necessary to make a blank determination with distilled water and lime water.

It is clear that in this blank determination the procedure must be identical with that used for the water, and that great care must be exercised to avoid carbonation of the lime water through exposure to the air. Twice the difference between the number of c.c. of $\frac{N}{80}$ sulphuric acid required to neutralize 100 c.c. of the lime water blank, and the number of c.c. of $\frac{N}{80}$ sulphuric acid required to neutralize the excess of lime in 100 c.c. of the sample, is an expression, in terms of $\frac{N}{80}$ sulphuric acid, for the amount of magnesium present in 100 c.c. of the water under examination. The following formula is convenient for calculating the amount of magnesium in parts per million.

Let S = Number of c.c. of $\frac{N}{80}$ H_2SO_4 required to neutralize 100 c.c. of the lime water blank.

Let N = Number of c.c. of $\frac{N}{80}$ H_2SO_4 required to neutralize the excess of calcium oxide in 100 c.c. of the mixture of lime water and of hard water.

Then Magnesium (Mg) Parts per million = $2.4 (2S - 2N)$.

The method gives very satisfactory results provided that the precautions mentioned above are rigidly observed. The change in the strength of the lime water during the heating involved in this method is illustrated by the following example of an analysis:

Strength of Lime Water (Available CaO).		
Parts per million.		
Lime Water Blank.		
Before Heating		After Heating
974		896
Analysis.		
Formula: $Mg = 2.4 (2S - 2N)$, = 2.4 (40-18) therefore		
$S = 20$	$N = 9$	= 53 parts per million.

A gravimetric analysis of the same water gave 55 parts magnesium per million. The above volumetric results agree very closely with the gravimetric results, and appear to be well adapted for the rapid determination of magnesium in hard waters.

Calculation of the Total Calcium.—The total hardness of a water expressed in terms of calcium carbonate, is the sum of the alkalinity and the incrustants, which are generally expressed in terms of calcium carbonate. If from the total hardness thus expressed an amount of calcium carbonate equivalent to the magnesium present be deducted, the remainder will, obviously, be an expression in terms of calcium carbonate, for the hardness due to calcium.

Incrusting Calcium.—The determination of the incrusting calcium consists in boiling 500 c.c. of the water in a porcelain dish to a volume of about 200 c.c.; removing the precipitated carbonates by filtration and determining the calcium in the filtrate by the usual gravimetric method. The amount of incrusting calcium is the difference between the total amount of calcium found to remain after boiling, and the amount of it due to dissolved normal carbonate of calcium. Comey states that the solubility of the normal carbonates consists of 20 parts calcium carbonate and of 17 parts magnesium carbonate per million. The correction to be applied is, obviously, in terms of calcium eight parts per million. (20×0.40 .)

CALCULATION OF THE ANALYSIS.

From the data listed above the analysis may be calculated. The results are expressed under two separate headings, namely, Alkalinity and Incrustants; and, as previously mentioned, the bases are expressed as such.

COMPONENT BASES OF THE ALKALINITY.

Calcium.—To compute the amount of calcium attributing to the alkalinity, that quantity present as an incrustant is subtracted from the total calcium.

Magnesium.—It is obvious that the difference between the alkalinity determined by titration, and that portion found to be due to calcium, must be an amount of calcium carbonate equivalent to the magnesium component of the alkalinity.

COMPONENT BASES OF THE INCRUSTANTS.

Calcium.—This constituent is determined directly by the method already described.

Magnesium.—The incrusting magnesium, by reasoning analogous to that used in the computation of the calcium contributing to the alkalinity, is the difference between the total magnesium and that constituting a part of the alkalinity.

Total Incrustants by Calculation.—The total incrustants in the water, expressed in terms of calcium carbonate, are clearly the sum of their component bases, calcium and magnesium, expressed in terms of calcium carbonate.

The following example is given as an illustration of the application of the foregoing procedures:

<i>Constituents</i>	<i>Parts per million</i>
Total Calcium (Ca)	= 98
Total Magnesium (Mg)	= 44
Alkalinity (CaCO_3)	= 252
Total Incrustants (CaCO_3)	= 174
Incrusting Calcium (Ca)	= 25

Calculation of the Component Bases of the Alkalinity.

<i>Calcium</i>	<i>(Parts per million)</i>	<i>Magnesium</i>	
Total Calcium	= 98	Alkalinity	= 252
Incrusting Calcium	= 25	Calcium Component	= 183
Calcium as alkalinity	= 73	Magnesium as CaCO_3	= 69
Equivalent Calcium Carbonate	= 183	Magnesium	= 17

Calculation of the Component Bases of the Incrustants.

<i>Calcium</i>	<i>(Parts per million)</i>	<i>Magnesium</i>	
Incrusting calcium (Ca)	= 25	Total magnesium (Mg)	= 44
Equivalent calcium carbonate	= 63	Mg component of alkalinity	= 17
Total incrustants computed	= 176	Incrusting Mg	= 27
Total incrustants by "Soda Reagent"	= 174	Equivalent calcium carbonate	= 113

To facilitate a comparison of this form of expressing an analysis with the older method of reporting data for water softening purposes, the above constituents are shown assembled in the following table:

<i>Alkalinity</i>		<i>Incrustants</i>	
Ca = 73		Ca = 25	
Mg = 17		Mg = 27	
Total alkalinity = 252		Total incrustants = 176	By "Soda Reagent"
Half-bound carbonic acid = 111			= 174 By Computation

THE THEORY OF THE ACTION OF LIME AND SODA ASH AS SOFTENING AGENTS FOR A MAGNESIUM WATER.

Lime and soda ash are of chief economic interest for water softening upon a large scale. The theory of their action upon a magnesium water is as follows:

Lime.—As is well known, the softening by lime of hard waters containing calcium alone, consists only in a neutralization of the free and the half-bound carbonic acid present. In the presence of magnesium, however, complications arise owing to the property of magnesium of forming soluble basic salts. For a magnesium water, such as was the water under discussion, a neutralization of the carbonic acid, converts the magnesium component of the alkalinity to a soluble basic carbonate, the removal of which is effected only by an additional amount of lime, which is that quantity sufficient to change the magnesium to the difficultly soluble hydrate. The incrusting magnesium must also be converted to hydrate since by the action of the soda ash subsequently added, this portion also of the magnesium is converted to a basic carbonate requiring an alkali for its precipitation. It is clear that the amount of lime required to soften a magnesium water is an amount sufficient to neutralize the carbonic acid, together with the additional amount necessary to convert to the hydrate form, the total quantity of magnesium present.

Soda ash.—Soda ash, as is clearly understood, precipitates the calcium component of the incrustants, and converts to a basic carbonate the magnesium component. Since the action of lime upon the incrusting magnesium results in the formation of an equivalent amount of a calcium incrustant, the bases of the incrustants may be considered as consisting of calcium alone, so that, in calculating the amount of soda ash required to treat a magnesium water similar to the one under consideration, the total amount of the incrustants may be taken as a basis.

It is apparent that magnesium requires for its removal from a hard water, an amount of lime twice as great as would an equivalent amount of calcium. And further, that to compute the amount of lime required, it is necessary to determine only the total amount of magnesium present in addition to the determination of the carbonic acid.

CONCLUSIONS.

The study of the problem of softening a hard water containing an unusually large relative amount of magnesium, has shown that the data required to calculate the quantities of lime and of soda ash necessary to soften it may be obtained without making a complete mineral analysis. The data are: The carbonic acid, the total magnesium and the incrustants. From the quantities of these constituents in the water, together with a determination of the amount of calcium contributing to the incrustants, there may be calculated the component bases of the alkalinity and the incrustants, respectively.

An assembled analysis of the water is readily computed from these data, and in the opinion of the writer, the statement of the analysis in terms of the bases present, is in sufficient detail. Such a method, obviously, avoids all assumptions as to the specific combinations of the bases and of the acid radicals present. A determination of the quantity of sulphuric anhydrid in the water is unnecessary when the calculation of the softening treatment is based on these data, and therefore the much disputed question of the apportionment of the sulphates and the bases would not appear to be a factor in water softening analyses, at least for conditions similar to those under discussion.

The writer wishes to acknowledge his obligations to Mr. George W. Fuller for invaluable advice and criticisms, and to Mr. George A. Johnson, under whose supervision the investigation was conducted, and with whose valuable counsel and cooperation this paper has been prepared.

TYPHOID-LIKE BACILLI IN THE WATER SUPPLY OF FREDERICTON, N. B.

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THE City of Fredericton, N. B., is situated on the south bank of the St. John River, which at this point is about three quarters of a mile wide, with little current. The tide does not come further up the river than Gagetown, about 35 miles below Fredericton, but the effect of the tide is felt at Fredericton, there being a rise of an inch at high water and five or six inches at low water.

The city is built on sandy soil with here and there layers or banks of clay. By digging down a few feet, water may be reached at any time in the year, and the water level rises and falls according as the season is wet or dry. Every spring many cellars are flooded.

The city has no system of sewerage; but to some slight extent the drains are used as sewers. These flow into the river at different points along the city front. Nearly every house has one or more cess pools. The hospital is situated 100 feet above the pumping station and 300 feet from the bank of the river. This bank is about 20 feet high and there is a gradual incline from the hospital to the edge of the bank. Formerly, there was a sewer from the hospital to a place below the intake; this, however, became plugged and for over a year past the drainage from the hospital has emptied into a hole just behind the hospital and from this receptacle it has drained into the river.

The water supply for the city is taken from the St. John River, 150 feet from the shore and directly opposite the pumping station.

The number of cases of Typhoid fever officially reported in Fredericton (population 7,000) during the last few years are as follows:

The year ending October 31, 1899, 71 cases.					
"	"	"	"	"	1900, 21
"	"	"	"	"	1901, 10

The year ending October 31, 1902, 33 cases.

“ “ “ “ “ 1903, 29 “

and the number of cases from November 1, 1903, to April 4, 1904, was 24. At the time of writing, there are 15 cases in the hospital and over 50 in the city. Besides the cases which are officially reported, the writer is informed that there are dozens each year which are cared for at home, and are not reported. The cases are from all parts of the city supplied with city water. Across the river at St. Mary's, where artesian well waters are used, there have been no cases of typhoid this year and none for several years past, except one or two and these among those who work in Fredericton and drink Fredericton water. From a careful examination of the distribution of the cases, dissemination through infected milk from one or more milk dealers may be excluded.

On March 15, 1904, the writer received from Fredericton two bottles of water, well packed and in good condition. One of these samples was taken from the river at a point one mile above the city. The second sample was made up of water from running taps in three different parts of the city.

The chemical analyses of these waters were as follows:

	River Water (parts per million)	City Water
Solids at 212° F.....	8.	72.
Solids after ignition	3.	32.
Loss on ignition.....	5.	40.
Free ammonia.....	0.09	0.015
Albuminoid	0.045	0.1875
Nitrogen in Nitrates and Nitrites	0.111	0.267
Phosphates	free	free
Chlorine	1.0	30.

The above analyses were made by Mr. F. T. Shutt, Chemist of the Experimental Farm at Ottawa. Commenting on the above analyses, he stated that "It is to be regarded as a suspicious sign that the differences in the analytical data are to be observed, and that between the point where the sample was taken in the river and the mouth of the intake pipe the quality of the water was, for some cause, changed for the worse."

The bacteriological analyses were as follows:

River Water:

Total number of bacteria per c.c., 141,000.

Liquefying colonies chiefly $\frac{B. fluorescens liquefaciens}{9000 \text{ per c.c.}}$.

Non-liquefying colonies, 132,000 per c.c.

Fermentation tubes containing two per cent glucose broth inoculated with $\frac{1}{4}$ c.c. of water gave no growth in the closed arm after 48 hours at 37° C.

Inoculated with $\frac{1}{2}$ c.c. water gave slight growth and cloudiness and seven per cent gas in 48 hours.

Carbolic broth (containing four c.c. of five per cent solution of carbolic acid in 100 c.c. of beef broth) inoculated with $\frac{1}{4}$ c.c. of river water and incubated at 37° C. gave no growth in 48 hours. Inoculated with $\frac{1}{2}$ c.c. water and incubated for 48 hours at 37° C., no growth.

Two guinea pigs were inoculated. One, intraperitoneally, with two c.c. of glucose broth from fermentation tube inoculated with $\frac{1}{2}$ c.c. water; and the other, intraperitoneally, with two c.c. of the carbolic broth culture inoculated with $\frac{1}{2}$ c.c. water. Both cultures had previously been incubated for 48 hours at 37° C.

The results were nil; the animals remained well and healthy.

City water:

Total number of bacteria per c.c., 34,650.

Liquefying colonies, principally $\frac{B. fluorescens liquefaciens}{3150 \text{ per c.c.}}$.

Non-liquefying colonies, 31,500 per c.c.

Of these, a number looked like colonies of the colon bacillus.

Agar plates incubated at 37° C. for 24 hours gave 16,150 colonies of bacteria per c.c. Colon bacilli were present in considerable numbers.

Fermentation tubes inoculated with $\frac{1}{4}$ c.c. water and incubated at 37° C. gave 20 per cent gas in 48 hours. Others inoculated with $\frac{1}{2}$ c.c. water gave 45 per cent gas in 48 hours.

Carbolic broth tubes inoculated with $\frac{1}{4}$ c. c. water and incubated at 37° gave considerable cloudiness in 24 hours.

Gelatin plates were made from the carbolic broth cultures

and these plates gave practically a pure culture of colon-like bacteria. These plates were carefully examined and 21 colonies, which seemed to resemble the growth of *B. typhosus* on gelatin, were isolated and these sub-cultures were passed through a number of differential tests to see if they resembled the typhoid or the colon bacillus. These tests will be given in detail later on.

Animal inoculations.—Two guinea pigs were inoculated with cultures obtained from the tap water.

One guinea pig was inoculated intraperitoneally with two c. c. of a 36-hour old glucose broth culture inoculated with $\frac{1}{2}$ c. c. water. Eight hours afterwards the animal appeared drowsy, with labored breathing, the hind legs were partly paralyzed, general malaise, and the animal died 24 hours later.

Postmortem made directly after death showed fibrinous serous peritonitis. Spleen and heart-blood contained bacteria resembling *B. coli*.

The other guinea pig, injected intraperitoneally with two c. c. of a 36-hour old carbolic broth culture inoculated with $\frac{1}{2}$ c. c. tap water showed the same symptoms as the animal inoculated with the glucose broth culture. It died in 36 hours. Postmortem appearances similar to above animal.

The 21 colonies isolated from gelatin plates, inoculated with carbolic broth, were put through a number of differential tests and compared with typical cultures of *B. typhosus* and *B. coli*. The results of these tests are given in tabular form, and a few explanatory remarks are necessary in connection therewith.

The surface colonies selected from the gelatin plates were those which grew more slowly and were thinner than the majority of the colonies on the plate; many of which showed the typical vine leaf markings and thicker and more rapid growth of *B. coli*.

Dextrose Broth.—This broth was made up from Liebig's extract of beef, one per cent of peptone and two per cent of dextrose. *B. coli* gave from 50 per cent to 75 per cent of gas in this medium. Nos. 1, 3, 4, 6, 10, 12, 14, 17, 19, and 20 produced gas in this medium, varying from 10 to 60 per cent.

Indol.—Dunham's solution was employed. Cultures 5 and 10 days old, incubated at 37° C., were used for this test. The typical

B. coli produced indol in abundance. There was no formation of indol by the other varieties.

Milk.—The milk cultures were incubated at 37° C., and held under observation for a month at this temperature. Nos. 4, 14, 16, and 20 coagulated. Sixteen and 20 produced considerable gas.

Potato.—*B. coli* produced a thick, yellowish-brown growth on the potatoes used, and numbers 1, 3, 4, 6, 9, 10, 12, 14, 17, 19, 20, and 21 also gave colored growths, in some cases not so marked in color or thickness as *B. coli*. The growths on Nos. 5 and 11 were very slightly tinged with color. There was a thin, transparent growth, scarcely visible to the naked eye, upon the surface of the other potatoes. These growths were submitted to a microscopical examination which showed the presence of large numbers of bacteria.

Litmus Whey, containing one per cent of peptone.—Cultures were incubated at 37° C. for 10 days. Reaction was tested on the 5th and on the 10th days. Nos. 4, 6, 14, 16, and 20 showed marked acidity. Most of them taking 30 per cent of $\frac{N}{10}$ alkali to neutralize. The remaining cultures wanted less than five per cent of $\frac{N}{10}$ alkali to neutralize the acidity.

Those varieties which showed most resemblance to *B. typhosus* were put through a few more differential tests with the following results:

Neutral Red Glucose Agar.—*B. coli* produced gas and a canary yellow fluorescence, and the remaining cultures showed growth, but no change in color and no production of gas.

Proskauer and Capaldi's Medium, No. I.—A culture of *B. typhosus* produced no growth or change in reaction in this medium. *B. coli* gave good growth with acid reaction. Nos. 1, 2, 7, 15, and 18 gave slight growth with acid reaction. Nos. 13 and 18 gave growth with acid reaction later changing to an alkaline reaction.

Proskauer and Capaldi's Medium, No. II.—Nos. 1, 2, 6, 7, 13, 15, 16 and 18 gave growth with acid reaction, resembling growth of the *B. typhosus* in this medium. *B. coli* gave growth with a neutral or faintly alkaline reaction of this medium.

Agglutination.—The antityphoid serum used for this was obtained through the courtesy of the Parke, Davis Company, of Detroit. It was a very powerful serum. The agglutinating limit of this serum with my *B. typhosus* was not worked out. In a dilution of 1:250,000, it gave a marked agglutination with this culture. For the purpose of testing the various varieties, four dilutions were made up—1:100,000, 1:10,000, 1:1,000, and 1:500. Four varieties, Nos. 8, 13, 15 and 18, gave complete agglutination in dilutions of 1:1,000 and 1:500, No. 2 gave complete agglutination with dilution 1:500.

A glance at these results will show that a number of these varieties were very closely allied to *B. typhosus* and showed greater resemblance to this organism than to the varieties described by Houston and Horrocks under the name of *B. typhosus simulans*. On the Proskauer and Capaldi's medium 1, the growth compared with *B. typhosus* was atypical.

A guinea pig inoculated with $\frac{1}{2}$ of an agar culture of variety No. 18 incubated for 24 hours at 37° died in 36 hours. The bacteria present in the spleen and heart blood were similar to the organism inoculated.

It seems unnecessary to comment upon the condition of the water supply of Fredericton. The drainage of the city and some of its sewage, the sewage from the hospital and the drainage from the cemetery, all empty into the St. John River close to the shore and these sources of contamination are both above and below the water intake and there seems to be sufficient tide in the river to contaminate the water *above* the intake, with the sewage which enters the river from *below* the pumping station.

NOTE.—The media used in this work were, unless otherwise stated, prepared according to the recommendations of the Laboratory Committee of the American Public Health Association.

All cultural tests were made in duplicate, some in triplicate.

VARIETY	GAS IN DEXTROSE BROTH	INDOL FORMATION	MILK	GROWTH ON POTATO	LITMUS WHEY	NEUTRAL RED AGAR	PROSKAUER-CAPALDI		REACTION WITH ANTI-TYPHOID SERUM			
							I	II	1:100,000	1:10,000	1:1,000	1:100
Typhoid Colon	0	0	0	0	0	0	No growth, no change in reaction. Growth, reaction acid in 24 hrs. Acid 4 days.	Growth, reaction strongly acid. Growth, reaction neutral or faint alk.	+	+	+	+
1	+	0	Coag. gas	+	+	0	Slight growth, reaction acid.	Growth, reaction strongly acid.	0	0	0	0
2	+	0	0	+	0	0	Slight growth, reaction acid.	Growth, reaction strongly acid.	0	0	0	0
3	+	0	0	+	0	0	Slight growth, reaction acid.	Growth, reaction strongly acid.	0	0	0	0
4	+	0	Coag. 2 days	+	+	0			0	0	0	0
5	+	0	0	+	+	0			0	0	0	0
6	+	0	0	+	+	0			0	0	0	0
7	+	0	0	+	+	0	Slight growth, reaction slightly acid.	Growth, reaction strongly acid.	0	0	0	0
8	0	0	0	0	0	0	Slight growth, reaction slightly acid.	Growth, reaction strongly acid.	0	0	0	0
9	+	0	0	+	+	0	Slight growth, reaction slightly acid.	Growth, reaction strongly acid.	0	0	0	0
10	+	0	0	+	+	0	Slight growth, reaction slightly acid.	Growth, reaction strongly acid.	0	0	0	0
11	+	0	0	+	+	0	Slight growth, reaction slightly acid.	Growth, reaction strongly acid.	0	0	0	0
12	+	0	0	+	+	0	Slight growth, reaction slightly acid.	Growth, reaction strongly acid.	0	0	0	0
13	0	0	0	0	0	0	Slight growth, reaction slightly acid.	Growth, reaction strongly acid.	0	0	0	0
14	+	0	Coag. 2 days	+	+	0	Slight growth, reaction slightly acid.	Growth, reaction strongly acid.	0	0	0	0
15	0	0	0	0	0	0	Slight growth, reaction slightly acid.	Growth, reaction strongly acid.	0	0	0	0
16	0	0	Coag. gas	0	+	0	Slight growth, reaction acid, later alkaline.	Growth, reaction strongly acid.	0	0	0	0
17	+	0	0	+	0	0	Growth, reaction acid.	Growth, reaction strongly acid.	0	0	0	0
18	+	0	0	+	0	0			0	0	0	0
19	+	0	0	+	0	0			0	0	0	0
20	+	0	Coag. gas	+	0	0			0	0	0	0
21	0	0	0	+	0	0			0	0	0	0

Gas in dextrose broth	+	= gas	Milk	0 = no change, no gas, no co- agulation	Neutral Red Agar	+	= canary yellow, fluorescence, gas
Indol formation in Dun- ham's Solution tested after 5 and 10 days incubation at 37° C.	+	= indol reaction	Potato	0 = invisible growth (trans- parent film)	Anti-Typhoid Serum	+	= no change in color, no gas
	0	= no reaction	Litmus Whey	+	= very slightly acid	+	= agglutination
				+	= marked acidity 10-30 per cent N alkali to neutral- ize	0	= no agglutination

THE PERSISTENCE OF AGGLUTINABILITY IN TYPHOID BACILLI IN WATER.

EDWIN O. JORDAN.

It has been established by the researches of Bail,¹ Walker² and Müller³ that the agglutinability of typhoid bacilli can be altered by gradual immunization to immune serum. Kirstein⁴ has also shown that it is possible to effect a slight increase or decrease in the agglutinability of certain races by subjecting the bacilli to various chemical and physical influences; change in agglutinability, however, was not great and a permanently inagglutinable race could not be produced.

The question of a possible loss of agglutinability in typhoid bacilli in sewage or water is one of practical importance, since in many instances recognition and identification of the typhoid bacillus have been made to hinge upon the positive outcome of the agglutination reaction.

Both observation and experiment bear upon this question. In the former category may be placed the observations of Remlinger and Schneider⁵ who found in water, soil and other situations bacteria that were said to resemble typhoid bacilli in every respect except that they failed to agglutinate with typhoid serum and were not pathogenic for animals. Remlinger and Schneider were tempted to conclude that these were typhoid germs that had parted with their agglutinability under the conditions to which they had been subjected.

Dealing with the same matter on the experimental side, Remy⁶ rejects the view advanced by Wathelet⁷ and Grimbert⁸ to explain the failure to isolate *B. typhosus* after a more or less prolonged association with *B. coli*. Wathelet and Grimbert believed that the typhoid bacillus, when sown together with *B. coli* "succumbs

¹ *Archiv f. Hyg.*, 1902, 42, p. 307.

³ *Munch. med. Wchnschr.*, 1903, 50, p. 56.

² *Jour. of Path. and Bact.*, 1902, 8, p. 34.

⁴ *Ztschr. f. Hyg.*, 1904, 46, p. 229.

⁵ *Ann. de l'Inst. Past.*, 1897, 11, p. 55.

⁶ *Ibid.*, 1900, 14, p. 705.

⁷ *Ibid.*, 1895, 9, p. 252.

⁸ *Compt. Rendu de la Soc. de Biol.*, 1894, 46, p. 399.

in the struggle." Remy maintains rather that the properties of the two organisms are profoundly modified by life in common. As one consequence of this association the typhoid bacillus, according to Remy's statements, may lose its sensibility towards the specific agglutinin. When grown separately in neutral peptone water both organisms retained their specific qualities. Horrocks¹ on the other hand, in a similar experiment, found that *B. typhosus* when grown with *B. coli* maintained its power of agglutinating with specific serum as long as it could be isolated from the mixture (13 days).

The observations of Remy and Horrocks thus stand in direct conflict. The practical importance of accumulating facts as to the frequency with which so significant a differential peculiarity as agglutination is destroyed by aquatic life or by association with *B. coli* leads the writer to record a few experiments on this point made in connection with a study of the longevity of typhoid bacilli in water.²

Experiment 1.—Two loopfuls of a 24-hour old agar culture of *B. typhosus* (Strain II) and one loopful of *B. coli* (Strain I) were introduced into 500 c.c. of sterile tap water in a glass flask and kept at room temperature for 12 days. Typhoid bacilli were then isolated by plating in litmus lactose agar, no difficulty being experienced in discovering typhoid colonies. Agglutination was tested macroscopically with immune serum from a rabbit, using the stock typhoid culture (Strain II) as a control. Both the stock culture and the culture obtained from the tap water agglutinated to exactly the same degree (1:500) with the typhoid serum. The colon bacilli that had been kept in the water with typhoid bacilli showed no agglutination whatever with a 1:40 dilution of the typhoid immune serum.

Result.—No change in agglutinability of the typhoid bacillus after association with *B. coli* in tap water for twelve days.

Experiment 2.—The conditions of this experiment were similar to those described in Experiment 1. The immune serum used in this instance agglutinated completely in 1:1000 dilution. The typhoid kept in stock (on agar) and the culture isolated after 18 days' sojourn in water together with *B. coli*, agglutinated in precisely the same way in a 1:200, 1:400, 1:1000 and 1:2000 dilution.

Result.—No change in agglutinability after association with *B. coli* in tap water for 18 days.

Experiment 3.—Five hundred centimetres of sterile sewage was inoculated with a mixture of *B. typhosus* and *B. coli* in the proportions given in Experiment 1. Immune rabbit serum agglutinating completely in 1:1000 dilution

¹ *Bacteriological Examination of Water*, 1901, p. 219.

² *Jour. Inf. Dis.*, 1901, 1, p. 641.

was used. Twenty days after inoculation *B. typhosus* was isolated from the mixture by the plate method of Hiss. This culture agglutinated exactly like the stock culture, when compared in 1:1000 dilution. The culture of *B. coli* derived from the mixture showed no agglutination in a 1:40 dilution of the serum.* Another flask of sewage was inoculated at the same time with a pure culture of *B. typhosus* and the organisms recovered after 20 days. In this case also, agglutinability was normal.

Result.—When inoculated into sterile sewage both alone and in association with *B. coli*, the typhoid bacillus retained its agglutinability unimpaired for 20 days.

Experiment 4.—Two loopfuls of a 24-hour old agar culture of *B. typhosus* (Strain X¹) and one loopful of *B. coli* (Strain IV) were introduced into 500 c.c. of sterile tap water in a glass flask and kept at room temperature. *B. typhosus* was isolated from the mixture after 34 days and was agglutinated by immune serum (Strain II) in 1:1000 dilution precisely like the control culture. *B. coli* from this flask showed no agglutination in 1:40 dilution of the serum.

Result.—No change in agglutinability after association with *B. coli* in tap water for 34 days.

From these experiments it appears (1) That the typhoid bacillus may be isolated without special difficulty after association with *B. coli* in tap water and sewage for as long as 34 days; (2) That under these conditions some strains of *B. typhosus* retain their property of agglutinability absolutely intact.

* In some interesting observations recorded by Horrocks (*Bacteriological Examination of Water*, 1901, p. 227) it was found that the varieties of *B. coli* isolated from typhoid dejecta showed much greater sensibility to agglutination with typhoid immune serum than the varieties of *B. coli* found in normal stools. If these results of Horrocks are confirmed they present some perplexing theoretical problems. Horrocks himself in subsequent experiments found that *B. coli* grown in the presence of "typhoid toxins and agglutinins" evinced no increased sensibility to agglutination with typhoid serum. In our own experiments, just cited, *B. coli* after association in water with *B. typhosus* showed no enhanced susceptibility to agglutination with typhoid serum. The observations of Horrocks do not necessarily require the interpretation that he has placed upon them.

¹ *Jour. Infect. Dis.*, 1904, 1, p. 643.

THE CHEMICAL AND BACTERIAL COMPOSITION OF THE SEWAGE DISCHARGED INTO BOSTON HARBOR FROM THE SOUTH METROPOLITAN DISTRICT.

WITH SPECIAL REFERENCE TO DIURNAL AND SEASONAL
VARIATIONS.

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INTRODUCTION.

IN any problem of sewage disposal the composition of the sewage to be treated is the first and most essential factor. A few haphazard analyses are not sufficient. Diurnal and seasonal variations must be determined and the total amount of organic matter to be handled estimated with some degree of accuracy. At the Sanitary Research Laboratory of the Institute of Technology we have based our ideas of the efficiency of our various experimental filters upon the studies outlined below—studies which included daily analyses extending over a period of nearly a year, and six special 24 series of hourly samples. With the exception of the Lawrence results, no fuller set of analyses has probably been made for any American city. We have thought therefore that a somewhat full discussion of the data might prove suggestive as to methods of approach, as well as of absolute value in defining the character of the Boston sewage studied.

STATISTICS OF CONTRIBUTORY AREA.

The entire metropolitan district of eastern Massachusetts covers nearly 200 square miles of territory and includes at present some 24 separate cities and towns. The sewage from this large area is discharged by two main sewers into Boston harbor, the region north of the Charles discharging continuously off Deer Island, while the sewage of the South Metropolitan District is stored in tanks at Moon Island and discharged into the harbor on the

ebb tide. (See map.) A third main sewer is now under construction which will relieve the Moon Island outlet, carrying the flow by gravity from the higher parts of the South Metropolitan District to a point of discharge farther out in the harbor. The South Metropolitan District, as at present constituted, includes the city of Boston (with the exception of East Boston, Charlestown, and the harbor islands); the cities of Newton, Waltham, and Quincy, and the towns of Brookline, Watertown, Milton, Hyde Park, and Dedham.

One large metropolitan interceptor receives the sewage from Newton, Waltham, Brookline, and Watertown, and another drains Milton, Quincy, Hyde Park, and Dedham. Both discharge into a main trunk sewer which receives directly most of the sewage of the city of Boston. Statistics for the metropolitan district as given in the reports of the Metropolitan Water and Sewerage Board omit all discharges entering the trunk sewer itself and not passing through one of the two metropolitan interceptors; but we have collected in Table 1 the data for the whole sewerage system discharging at Moon Island, combining with the figures given in the second annual report of the Metropolitan Water and Sewerage Board of January 1, 1903, statistics obtained from the office of the sewer division of the street department of the city of Boston, through the courtesy of Mr. E. S. Dorr, engineer in charge. The present total population (1903), is calculated by geometrical ratio from the censuses of 1895 and 1900; and the contributing population is estimated by assuming that it bears to the total population the same relation which the number of connections bears to the total number of dwelling-houses as stated in the assessor's records.

The Sewage Experiment Station of the Massachusetts Institute of Technology is situated at 786 Albany Street, Boston, at the point indicated by a star on the map. A $2\frac{1}{2}$ inch pipe connects the station with the trunk sewer of the South Metropolitan District just below the junction of the Albany Street interceptor and above the junctions of the South Boston and Dorchester intercepting sewers, into which not only those districts and a small portion of West Roxbury, but Milton, Hyde Park, Dedham,



TABLE 1.

STATISTICS OF THE SOUTH METROPOLITAN SYSTEM DISCHARGING AT MOON ISLAND.

Districts	Miles of Local Sewer Connected	Area Served Sq. Mi.	Number of Connections with Local Sewers	Estimated Present Total Population	Estimated Population Now Contributing Sewage	Estimated Number of Persons Served by Each House Connection
Boston—						
Wards 6, 7, 8, 9, 10, 11, 12..	64.18	3.60	15,562	169,200	169,200	10.8
South Boston—						
Wards 13, 14, 15.....	45.52	1.92	5,287	64,700	47,830	9.1
Roxbury—						
Wards 17, 18, 19, 21 (22)..	96.76	3.79	6,731	121,900	63,570	9.4
West Roxbury—						
Wards (22), 23.....	85.80	12.49	3,356	41,400	24,730	7.4
Dorchester—						
Wards 16, 20, 24.....	185.15	8.64	8,845	98,400	70,380	8.0
Brighton—						
Ward 25.....	59.60	4.28	2,405	22,400	14,350	6.0
Brookline.....	53.61	6.81	2,758	23,000	19,000	6.9
Newton.....	94.85	18.03	4,569	37,200	26,900	5.9
Watertown.....	30.41	4.04	1,467	10,700	7,900	5.4
Waltham.....	38.25	13.63	2,594	25,500	22,050	8.5
Milton.....	3.39	12.59	78	7,300	430	5.5
Hyde Park.....	17.11	4.57	609	14,100	5,360	8.8
Dedham.....	3.32	9.40	17	7,200	95	5.0
Quincy.....	35.93	12.56	952	26,500	5,300	5.6
Totals.....	813.88	116.35	55,210	669,500	577,065	8.1

TABLE 2.

STATISTICS OF THE AREA ABOVE THE SEWAGE EXPERIMENT STATION.

Districts	Miles of Local Sewer Connected	Area Served	Number of Connections with Local Sewers	Estimated Present Total Population	Estimated Population Now Contributing Sewage	Estimated Number of Persons Served by Each House Connection
Boston—						
Wards 6, 7, 8, 9, 10, 11, 12..	64.18	3.60	15,562	169,200	169,200	10.8
Roxbury—						
Wards 17, 18, 19, 21 (22)..	96.76	3.79	6,731	121,900	63,570	9.4
West Roxbury—						
Wards (22), 23.....	81.88	3.57	3,221	39,800	23,800	7.4
Brighton.....	59.60	4.28	2,405	22,400	14,350	6.0
Brookline.....	53.61	6.81	2,758	23,000	19,000	6.9
Newton.....	94.85	18.03	4,569	37,200	26,900	5.9
Watertown.....	30.41	4.04	1,467	10,700	7,900	5.4
Waltham.....	38.25	13.63	2,594	25,500	22,050	8.5
Totals.....	519.54	57.75	39,407	449,700	346,770	8.9

and Quincy discharge. The statistics for the area contributing the sewage actually analyzed are shown in Table 2. The population served appears to be about 350,000, a little more than one-half the total for the South Metropolitan District.

CALCULATION OF THE FLOW.

The first question to be settled concerned the variations in the sewage to be treated at different hours of the day and night; and in order to measure this factor we have made six sets of hourly analyses at different seasons of the year and under various weather conditions. In order to obtain an average analysis of the sewage flowing for 24 hours either one of two methods may be employed. Samples may be taken at short intervals, the volume of each sample being proportionate to the amount of sewage flowing at the time. Then if all the samples be mixed the result is a true average of the whole flow. A second and more convenient method is to take samples at regular intervals during the test and analyze each one separately. Then it is possible to calculate a "weighted average" by multiplying each analysis by the flow at the time of sampling, adding up the results and dividing by the total flow. Either method necessitates some knowledge of the hourly variation in the sewage flow.

We were unable to make meter measurements of the flow of sewage during the 24-hour runs, and it was impossible to make other gaugings on account of the size and the flat grade of the sewer. We were therefore forced to fall back upon indirect computations, which, though rough, possess sufficient comparative value for the weighting of hourly analyses.

The total length of the sewer between our intake and the pumping station at Dorchester is 11,514 feet. The sewer is circular in section, the diameter of the upper 6,874 being 9 feet, and of the remainder 10 feet 6 inches; it was built to a uniform slope of 1 in 2,500. At the point at which the sewer is enlarged from 9 feet to 10 feet 6 inches in diameter there enters from the north the South Boston interceptor, a sewer of circular section 6 feet in diameter, and at a point about 1,000 feet below this there enters from the south the Dorchester interceptor of

5 feet circular section. In the office of the Boston Main Drainage Works, on Massachusetts Avenue, directly opposite the point at which our intake enters the sewer, is installed an automatic gauge which records upon a revolving drum the elevation of the sewage. The pen of this indicator is operated by a float in a well, the latter being connected by a six-inch pipe with the sewer. At the pumping station in Dorchester a similar gauge is in operation at a point just above the screens. The permanent records of these gauges are preserved by the Sewer Division, to whose courteous officials we are indebted for their use and for other valuable assistance. We have also been allowed to examine and make use of the pump-log kept at the Dorchester station. We have therefore, for use in calculating the flow past our intake during any hour, the total amount pumped during that time, and the depth of the sewage at each end of the system at the beginning and end of the hour.

Considering the system below us as a reservoir we have a known amount flowing out (the amount pumped), and a known change in level from which we can calculate the amount flowing in. The calculation is complicated by the presence of the two intercepting sewers previously mentioned which enter the system below us; but one of these enters at too high a grade to affect the storage capacity, and the other stands full for too long a distance back materially to influence it. We shall therefore use in calculating our weighted analyses of sewage for 24 hours the total flow in the sewer as measured by the pump records, corrected for the storage in the system as indicated by the change in level. In comparing our monthly averages of daily analyses with one another we shall multiply each set by the total flow for the month.

In calculating the hourly flow we have first taken the recorded depth at each end of the system at the beginning and at the end of the hour, and calculated the volume of the layer of sewage included between these two surfaces. We have assumed the surfaces to be plane and taken as the cross-section of the layer of sewage the cross-section of the middle-point. The whole volume had to be divided into two parts, one in the nine-foot sewer and the other in the ten-foot-six sewer. The maximum observed

hourly variation in the amount of sewage in the sewer was but 10 per cent of the amount pumped in one hour and was generally less than two per cent. It was therefore necessary to calculate the storage value with an accuracy of only 10 per cent to avoid

TABLE 3.
FLOW OF SEWAGE — MILLION GALLONS PER HOUR.

TIME	July 1903 22 23	Aug. 1903 13 14	Nov. 1903 16 17	Dec. 1903 22 23	Feb. 1904 17 18	April 1904 12 13
9-10 A. M.	5.65	6.54	6.06	4.57
10-11 "	5.64	4.86	6.20	5.35	5.96	4.57
11-12 "	5.63	4.57	5.98	4.57	4.45	4.57
12- 1 P. M.	5.78	4.86	5.96	4.27	4.45	4.57 4.57
1- 2 "	5.65	4.71	5.96	4.57	5.96	4.57 4.57
2- 3 "	5.64	4.66	5.96	5.96	5.96	4.57 4.57
3- 4 "	5.44	4.83	5.96	5.96	5.96	4.57 4.57
4- 5 "	5.42	4.78	6.38	5.96	5.96	4.57
5- 6 "	5.42	4.68	6.07	5.96	5.96	4.57
6- 7 "	5.68	4.27	5.96	5.96	5.96	4.57
7- 8 "	7.23	4.53	5.96	5.96	5.96	4.57
8- 9 "	5.96	4.40	5.96	5.96	5.96	4.57
9-10 "	5.96	4.37	5.96	5.93	5.96	4.57
10-11 "	5.96	4.31	5.96	5.93	5.96	4.57
11-12 "	5.96	3.88	5.96	5.93	5.96	4.57
12- 1 A. M.	5.96	4.57	5.96	5.93	5.96	4.57
1- 2 "	5.96	4.46	5.96	5.65	5.96	4.57
2- 3 "	5.55	4.45	5.96	5.65	5.96	4.57
3- 4 "	5.55	4.57	5.96	5.85	5.92	4.57
4- 5 "	5.46	4.45	5.96	5.85	5.92	4.57
5- 6 "	5.83	4.50	5.96	5.85	5.92	4.57
6- 7 "	3.35	4.69	5.96	5.85	5.91	4.57
7- 8 "	4.86	4.45	5.96	5.85	5.90	4.57
8- 9 "	4.96	4.84	5.96	6.37	6.04	4.57

an error in the final result greater than one per cent. These calculated changes in the volume of the sewage from hour to hour were then applied as corrections to the quantities pumped as calculated from the pump records. If the height of sewage increased during the hour the correction was added; if it decreased, the correction was subtracted. In Table 3 are given the resulting hourly total flows into the system for the six days on which

we make our tests. They represent the flow past our intake plus the amount of sewage entering the system by the two intercepting sewers previously mentioned.

With regard to the absolute amount of sewage flowing past Albany Street the following data are available:

Messrs. R. G. Hartshorne and L. T. Howard, M. I. T., '04, carried out as a graduating thesis a study entitled "Measurement of the Flow in the Main Intercepting Sewer of Boston." This

TABLE 4.
FLOW OF SEWAGE IN MOON ISLAND SEWER AT ALBANY STREET.
(Hartshorne and Howard).

DATE	TIME	MEASUREMENTS			PUMP RECORDS CUBIC FEET PER SECOND	RATIO
		Mean Velocity	Area	Cubic Feet Per Second		
P.M.						
April 18.....	1:53- 2:50	2.04	57.2	116.8	222.9	.52
18.....	2:55- 3:51	2.12	58.6	124.0	222.9	.55
20.....	3:00- 4:14	1.93	63.6	122.8	225.4	.54
25.....	2:17- 3:26	2.17	55.3	120.0	226.9	.53
25.....	3:34- 4:37	2.11	55.6	117.0	226.9	.52
A.M.						
May 5.....	8:54- 9:46	1.41	63.6	89.9	164.7	.55
5.....	9:56-10:45	1.60	63.6	101.7	165.0	.62
14.....	7:40- 7:59	3.20	37.3	119.3	222.4	.54
14.....	8:04- 8:31	3.26	38.2	124.5	218.6—227.9	.54
14.....	8:47- 9:04	3.38	40.7	131.8	228.3	.60
						55.1

investigation was made under the direction of the late Mr. K. S. Sweet, Instructor in Civil Engineering, whose untimely death we, who knew him, still mourn. Messrs. Hartshorne and Howard have permitted us to use their figures comparing the results of meter measurements made in the sewer at a point just below our intake with the total amount pumped as obtained from the pump-logs of the sewer division. In making these measurements a large Haskell meter was used, with a long pitch wheel and electrical connections with a buzzer. A system of multiple point measurements was adopted. By use of a stop watch the time required to make a certain number of revolutions, either 10 or 20, was noted. In this way any clogging of the meter could be

instantly detected and remedied. Eight per cent was the maximum error allowed for in the calculation, but the actual error was undoubtedly much less. The following table, based upon the figures obtained, is calculated to show the ratio between the flow past our intake and the total amount pumped.

The results shown in Table 4 indicate a remarkably constant ratio between the amount flowing at Albany Street and the pumpage at the end of the sewer, a ratio of 50 to 60 per cent. The residue must be accounted for by slip of the pumps and by the contribution of the lower intercepting sewers. The concordance of the results obtained in the measurements seem to us to warrant the assumption that the sewage flowing by Albany Street represents from 50 to 60 per cent of the total entering the Moon Island sewer. At any rate the contribution of our sewer appears to be a constant factor of the whole.

METHODS OF SAMPLING AND ANALYSIS.

In the first (July, 1903) series of analyses samples were taken from the petcock of the pump, but the difficulty of obtaining anything like a representative sample in this way is very great. In the later runs our pump was run continuously for the 24 hours, pumping at a rate of about 1500 gallons per hour. Some two-thirds of this amount was wasted, the remainder being delivered into a tank of 540 gallons capacity at such a rate that it was nearly filled in one hour. At each hour the sewage was diverted into a second tank, that in the first tank was thoroughly mixed by stirring, and a half gallon sample bottle was filled from the tank by means of a cock at its mid-depth. The bottle was filled rather slowly and the sewage kept well stirred during the operation. Each sample therefore represents very closely the average composition of the sewage flowing during one hour. The advantage of this procedure is clearly indicated by the smoothness of the curves on diagrams II-VI as compared with diagram I. Analyses were made immediately after the collection of the sample.

The following determinations were carried out on each sample: free and albuminoid ammonia, nitrites, oxygen consumed, chlorine

and dissolved oxygen. The samples were also examined bacteriologically and the following groups of organisms counted; on lactose gelatin at 20° C. the total number of organisms, the number of liquefiers and of acid formers; on lactose agar at 37° the total number and the number of acid formers; and on agar at 20° the number of facultative anaërobes.

The chemical analyses were made according to the methods now generally employed in this country as described for example by Richards and Woodman (1904.) We have used permanent standards for the ammonia readings as suggested by Jackson (1900). All ammonia and nitrite values are reported in terms of nitrogen, and all the chemical results are expressed in parts per million. We have adopted the plan of reporting the dissolved oxygen in parts rather than in per cent of saturation. While the latter method of expression may have had some significance in the study of surface waters it has no value whatever in connection with sewage work or with filtration work in general. Owing to a change in the temperature of the water during treatment the results expressed in per cent of saturation may show an increase in oxygen while there is in reality a marked decrease. For the oxygen consumed determination we used the Kübel method, boiling for two minutes.

In the bacteriological work we have followed the methods proposed by the Committee on Standard Methods of the American Public Health Association. The anaërobic counts were made in the earlier part of the work after the method of Wright (1901), later by a method proposed by Rickards (1904). Both methods are the same in principle, the oxygen being absorbed by pyrogallate of potassium.

GENERAL RESULTS OF THE EXPERIMENTS.

The analyses reported in Tables 5-10 represent six sets, each of 24 hourly samples. The first two series, of July and August, 1903, were intended to be typical of summer conditions. During the July run heavy rain fell while the August run was in fair weather. The third series in November represents late autumn conditions, and the fourth and fifth in December and

DIAGRAM II.

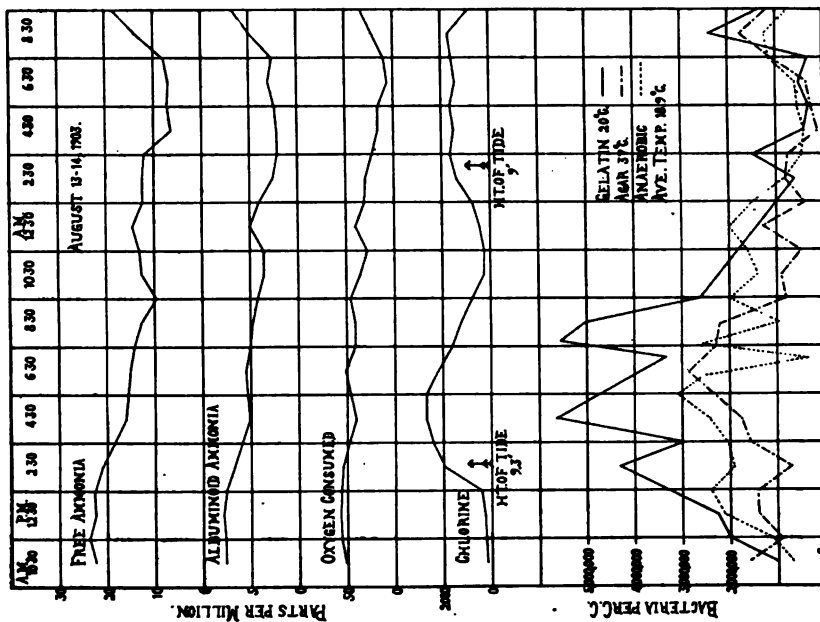


DIAGRAM I.

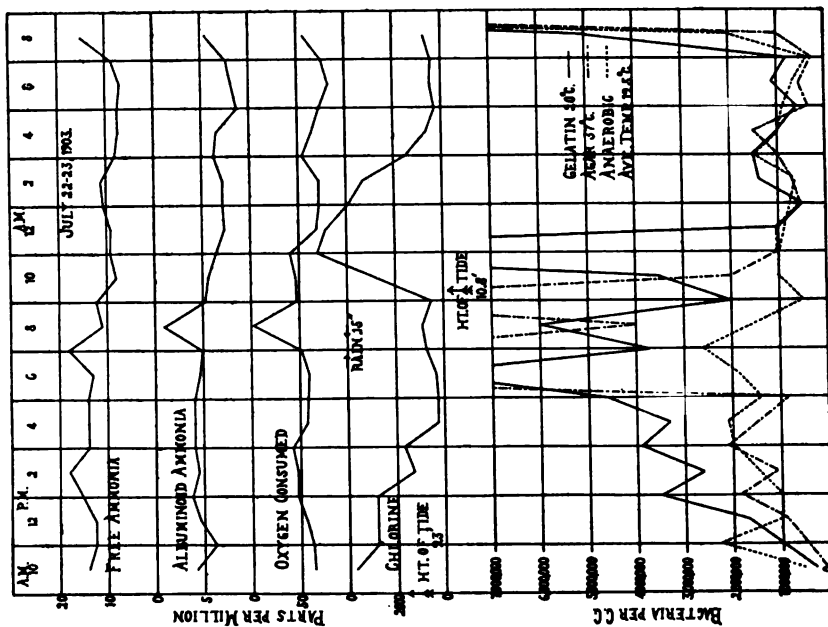


DIAGRAM IV.

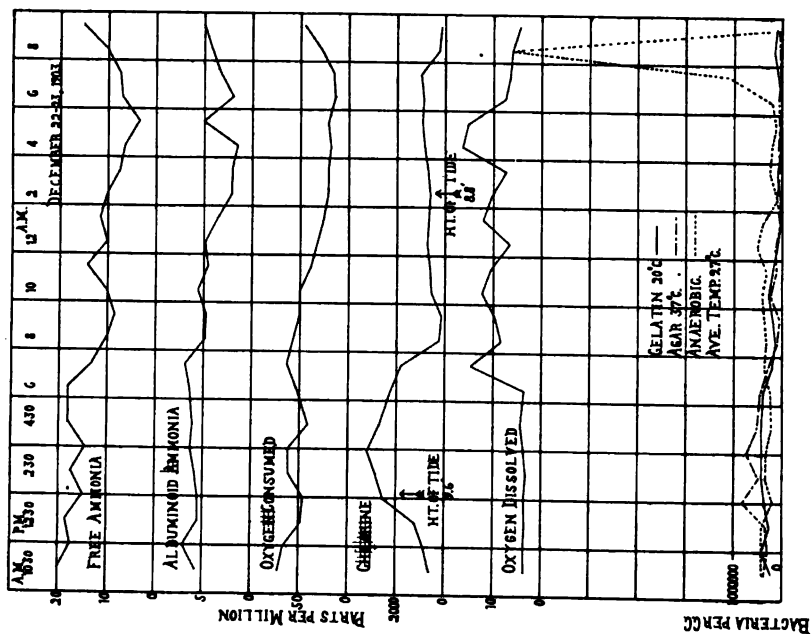


DIAGRAM III.

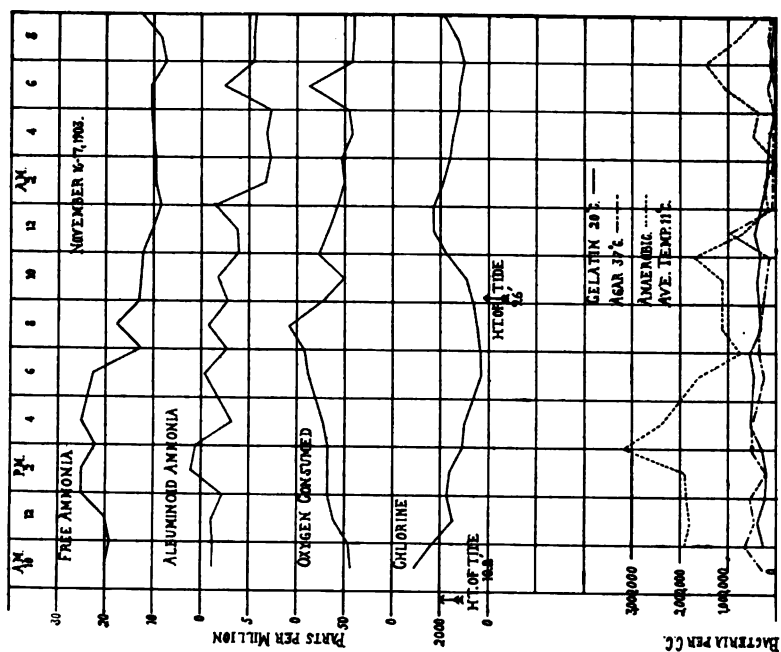


DIAGRAM VI.

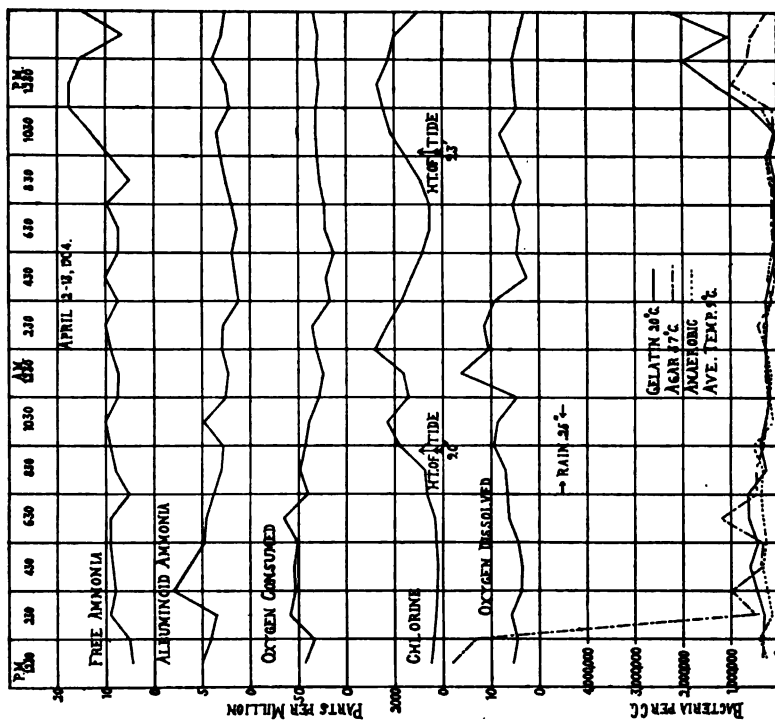


DIAGRAM V.

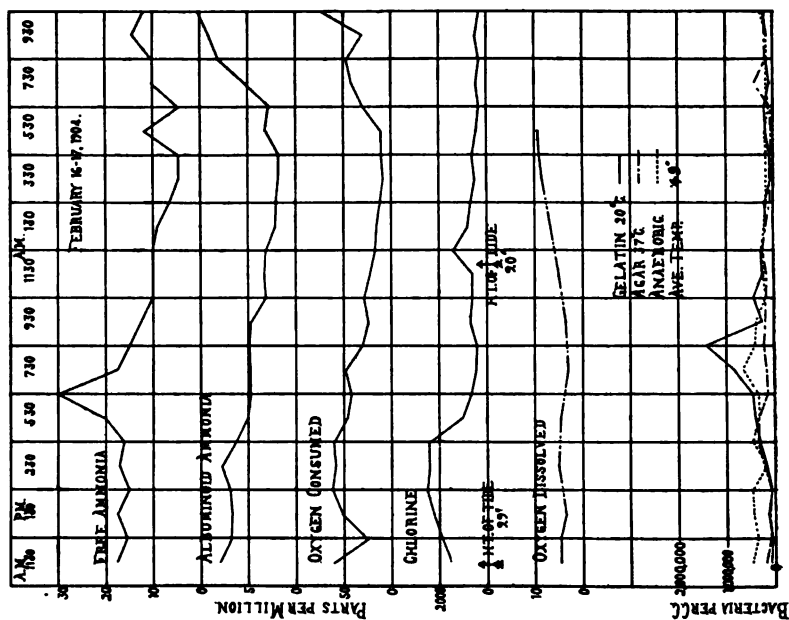


TABLE 5.

Parts per Million.

No.		NITROGEN AS *					OXYGEN CONSUMED	OXYGEN DISSOLVED	CHLORINE	NUMBER OF BACTERIA IN ONE CUBIC CENTIMETER						
		Free Am- monia	Albuminoid Ammonia		Ni- trates	Ni- trites				Lactose Gelatin at 20°			Lactose Agar at 37°		Anaerobic Lactose Agar at 20°	
			Total	Solu- tion						Sus- pen- sion	Acid	Liquefiers	Total	Acid		Total
	July 22	14.0	5.7	3.8	1.9	0.08	0.62	36	3,700	50,000	—	300,000	—	100,000	500,000	
33	10 A. M.	12.5	3.7	3.5	0.2	0.08	—	38	2,800	50,000	—	1,000,000	100,000	500,000	2,300,000	
34	11	12.5	5.3	2.8	2.5	0.10	—	44	2,800	250,000	200,000	1,700,000	350,000	950,000	1,000,000	
35	12	15.0	6.1	2.7	3.4	0.08	0.83	52	2,800	850,000	150,000	3,500,000	1,100,000	1,850,000	1,000,000	
36	1 P. M.	18.0	5.5	3.4	2.1	0.11	—	52	1,300	550,000	150,000	2,600,000	500,000	1,050,000	1,500,000	
37	2	15.0	5.8	3.4	2.1	0.10	—	57	1,700	400,000	200,000	3,900,000	650,000	2,100,000	2,000,000	
38	3	14.0	5.8	3.4	2.4	0.10	—	57	1,700	400,000	200,000	3,900,000	650,000	2,100,000	2,000,000	
39	4	14.0	5.8	2.0	3.8	0.10	1.03	42	280	300,000	—	3,300,000	100,000	12,000,000	2,100,000	
40	5	14.0	5.8	2.3	3.5	0.14	—	41	260	550,000	150,000	4,600,000	300,000	800,000	1,400,000	
41	6	13.0	5.2	2.8	2.4	0.16	—	40	360	1,000,000	150,000	8,800,000	100,000	17,000,000	1,900,000	
42	7	18.0	5.0	2.8	2.2	0.17	0.53	47	678	400,000	—	3,700,000	1,300,000	9,200,000	2,600,000	
43	8	11.0	9.0	2.2	6.8	0.10	—	98	871	2,000,000	1,000,000	6,000,000	1,500,000	4,000,000	—	
44	9	13.0	4.7	1.9	2.8	0.07	—	52	543	—	—	2,000,000	2,500,000	12,500,000	500,000	
45	10	8.0	4.4	2.2	2.2	0.08	0.48	53	2,840	—	—	3,500,000	500,000	2,000,000	1,000,000	
46	11	9.0	3.5	1.2	2.3	0.13	—	58	5,100	10,000,000	1,000,000	15,000,000	500,000	1,000,000	1,000,000	
47	12	9.0	2.6	1.4	1.2	0.12	—	31	4,890	—	—	1,000,000	—	1,000,000	—	
	July 23	10.5	2.8	1.4	1.4	0.13	0.78	28	3,980	—	—	500,000	—	500,000	—	
48	1 A. M.	11.0	2.8	1.3	1.5	0.05	—	26	3,330	200,000	200,000	1,400,000	200,000	650,000	650,000	
49	2	8.0	3.6	0.8	2.8	0.15	—	45	1,500	—	—	1,500,000	—	1,000,000	1,500,000	
50	3	7.2	3.4	0.9	2.5	0.15	0.53	35	632	—	—	1,000,000	—	1,500,000	1,000,000	
51	4	7.0	1.3	0.8	0.5	0.17	—	26	280	200,000	100,000	600,000	150,000	350,000	900,000	
52	5	7.2	1.9	1.1	0.8	0.15	—	18	440	200,000	150,000	1,100,000	150,000	550,000	700,000	
53	6	7.0	2.4	1.6	0.8	0.20	2.03	24	440	200,000	50,000	800,000	150,000	300,000	400,000	
54	7	8.8	2.4	1.6	0.8	0.20	2.03	24	720	400,000	300,000	5,000,000	400,000	1,000,000	2,000,000	
55	8	15.0	4.5	2.4	2.1	0.10	—	44	—	5,900,000	2,000,000	40,250,000	2,400,000	17,700,000	16,300,000	
56	9	—	—	—	—	—	2.53	—	—	—	—	—	—	—	—	
Averages.....		11.7	4.3	2.1	2.2	0.11	1.09	43	1,805	1,400,000	414,000	4,710,000	682,000	3,733,000	2,010,000	
Weighted averages		11.8	4.4	2.1	2.3	0.12	0.98	45	2,100	1,370,000	428,900	4,620,000	701,500	3,000,000	1,978,000	

TABLE 6.

Parts per Million.

No.	TIME	NITROGEN AS					OXYGEN CONSUMED	OXYGEN DISSOLVED	CHLORINE	NUMBER OF BACTERIA IN ONE CUBIC CENTIMETER						
		Free Ammonia	Albuminoid Ammonia			Nitrates				Lactose Gelatin at 20°				Lactose Agar at 37°		Anaerobic Lactose Agar at 20°
			Total	Solution	Suspension					Acid	Liquefiers	Total	Acid	Total		
August 13																
128	9:30-10:30	22.5	7.7	4.8	2.9	0.16	0.63	—	234	200,000	200,000	1,000,000	600,000	1,600,000	700,000	
129	10:30-11:30	24.0	7.7	4.7	3.0	0.14	—	—	236	700,000	100,000	2,000,000	450,000	900,000	1,150,000	
130	11:30-12:30	22.5	7.8	4.7	3.1	0.14	—	—	280	700,000	100,000	2,300,000	900,000	1,400,000	2,150,000	
P. M.																
131	12:30-1:30	22.5	7.7	4.4	3.3	0.14	—	420	—	1,200,000	400,000	3,300,000	300,000	1,400,000	2,400,000	
132	1:30-2:30	21.0	6.7	3.2	3.5	0.06	0.45	55	1,920	1,000,000	350,000	4,350,000	600,000	700,000	1,800,000	
133	2:30-3:30	18.0	5.7	2.8	2.9	0.08	—	48	2,430	1,050,000	50,000	2,900,000	450,000	1,600,000	2,050,000	
134	3:30-4:30	16.0	5.0	2.7	2.3	0.10	—	41	2,550	2,900,000	500,000	5,700,000	600,000	1,750,000	2,450,000	
135	5:30-6:15	15.0	5.4	2.7	2.7	0.10	—	51	2,160	1,150,000	300,000	4,100,000	1,150,000	2,900,000	2,500,000	
136	6:15-7:00	14.5	5.1	2.8	2.3	0.10	0.43	48	1,830	1,500,000	300,000	3,200,000	600,000	1,900,000	800,000	
137	7:00-7:45	14.0	5.0	2.2	2.8	0.08	—	43	1,560	—	—	5,600,000	1,200,000	2,350,000	2,600,000	
138	7:45-8:30	13.0	4.9	3.1	1.8	0.08	—	42	1,200	1,150,000	250,000	5,020,000	1,600,000	2,200,000	900,000	
139	8:30-9:30	9.5	4.3	4.1	1.9	0.10	—	760	760	750,000	250,000	2,650,000	400,000	750,000	2,000,000	
140	9:30-10:30	12.5	3.4	5.0	0.9	0.12	0.55	34	220	—	—	—	300,000	950,000	1,400,000	
141	10:30-11:30	13.0	3.5	2.0	1.5	0.08	—	27	220	—	100,000	1,800,000	200,000	450,000	1,600,000	
142	11:30-12:30	14.5	4.9	1.9	3.0	0.08	—	40	380	—	—	—	700,000	1,300,000	2,000,000	
August 14																
143	12:30-1:30	12.5	3.7	1.9	1.8	0.10	—	28	760	—	—	—	250,000	350,000	1,500,000	
144	1:30-2:30	12.5	2.4	0.9	1.5	0.12	0.40	29	1,420	50,000	—	600,000	100,000	700,000	800,000	
145	2:30-3:30	12.0	2.0	1.5	0.5	0.14	—	21	1,600	—	—	1,500,000	200,000	700,000	450,000	
146	3:30-4:30	6.5	2.0	1.4	0.6	0.14	—	17	1,520	—	—	400,000	—	100,000	400,000	
147	4:30-5:30	7.0	2.2	1.5	0.7	0.16	—	16	1,540	—	—	300,000	200,000	300,000	550,000	
148	5:30-6:30	7.0	3.1	2.4	0.7	0.12	0.75	8	1,480	50,000	50,000	500,000	350,000	400,000	600,000	
149	6:30-7:30	8.0	2.5	2.0	0.5	0.14	—	15	1,560	—	—	300,000	200,000	1,100,000	1,100,000	
150	7:30-8:30	14.0	5.2	2.6	2.6	0.14	—	22	1,670	300,000	100,000	2,400,000	200,000	1,750,000	1,250,000	
151	8:30-9:30	18.5	6.7	3.9	2.8	0.16	—	34	954	400,000	400,000	1,850,000	450,000	1,150,000	700,000	
Averages																
Weighted averages																
		14.6	4.8	2.7	2.1	0.11	0.54	37	1,206	873,000	223,000	2,465,000	519,000	1,195,000	1,410,000	
		14.7	4.8	2.7	2.1	0.11	0.64	37	1,213	860,000	232,000	2,480,000	520,000	1,220,000	1,415,000	

TABLE 7.

Parts per Million

No.	Time	NITROGEN AS					OXYGEN CONSUMED	OXYGEN DISSOLVED	CHLORINE	NUMBER OF BACTERIA IN ONE CUBIC CENTIMETER						
		Free Am- monia	ALBUMINOID AMMONIA			Ni- trates				Ni- trates	Lactose Gelatin at 20°			Lactose Agar at 37°		Anaerobic Lactose Agar at 20°
			Total	Solu- tion	Sus- pen- sion						Acid	Lique- fiers	Total	Acid	Total	
November 16																
428	9:00-10:00 10:00-11:00 11:00-12:00	20.0 19.0 20.0	8.8 8.5 8.8	3.4 4.0 4.2	5.4 4.5 4.6	0.14 0.80 8.80	— — —	44 46 62	2.30 1.50 1.40	3,050 2,400 1,560	— 50,000 60,000	0 0 0	280,000 400,000 400,000	270,000 680,000 440,000	— 1,910,000 1,810,000	
430	12:00-1:00 1:00-2:00 2:00-3:00	25.0 25.0 22.0	7.7 11.0 10.5	4.8 4.2 4.0	2.9 6.8 6.5	0.90 0.85 0.68	— — —	67 67 68	1.10 1.30 0.70	1,750 1,670 1,100	30,000 20,000 60,000	0 0 0	230,000 210,000 300,000	500,000 230,000 520,000	1,870,000 1,910,000 3,160,000	
434	3:00-4:00 4:30-5:50 7:00	25.0 22.5 12.5	6.7 9.5 7.3	4.8 4.2 3.4	1.9 5.3 3.9	0.10 0.80 0.00	— — —	72 88 93	1.00 340 1.30	1,000 340 380	100,000 20,000 70,000	0 0 0	550,000 470,000 540,000	460,000 280,000 320,000	2,410,000 1,650,000 750,000	
437	7:00-8:00 8:00-9:00 9:00-10:00	17.5 13.0 12.5	9.3 7.3 8.3	2.4 2.4 2.8	6.9 4.9 5.5	0.02 0.00 0.01	— — —	108 74 50	— — —	440 600 900	50,000 40,000 40,000	0 0 0	310,000 360,000 390,000	260,000 280,000 210,000	1,120,000 1,120,000 1,120,000	
440	10:00-11:00 11:00-12:00	12.0 10.0	6.0 6.1	2.3 1.9	3.7 4.2	0.00 0.00	— —	77 66	— —	1,780 2,300	30,000 80,000	0 0	330,000 450,000	140,000 960,000	1,710,000 680,000	
November 17																
442	12:00-1:00 1:00-2:00 2:00-3:00	8.5 9.3 9.5	8.5 3.3 2.9	5.5 1.3 1.3	3.0 2.0 1.6	0.00 0.00 0.00	— — —	57 50 53	— — —	2,300 1,900 1,600	20,000 20,000 40,000	0 0 0	330,000 190,000 160,000	80,000 60,000 50,000	160,000 250,000 30,000	
445	3:00-4:00 4:00-5:00 5:00-6:00	10.0 10.5 10.5	3.0 2.6 7.6	1.3 .9 1.5	1.7 1.7 6.1	0.00 0.00 0.10	— — —	43 45 88	— — —	1,520 1,280 1,100	20,000 — —	0 0 0	130,000 40,000 120,000	60,000 60,000 190,000	460,000 360,000 1,070,000	
448	6:00-7:00 7:00-8:00 8:00-9:00	7.5 8.5 12.5	4.5 4.5 4.3	1.3 1.9 2.0	3.2 2.6 2.3	0.10 0.60 0.80	— — —	43 41 41	— — —	1,020 1,260 1,860	40,000 — 30,000	0 0 0	80,000 160,000 270,000	60,000 170,000 288,000	1,460,000 830,000 340,000	
Averages.....																
Weighted averages																

TABLE 8.

Parts per Million

No.	TIME	NITROGEN AS						OXYGEN CONSUMED	OXYGEN DISSOLVED	CHOLINE	NUMBER OF BACTERIA IN ONE CUBIC CENTIMETER						
		Free Am- monia	ALBUMINOID AMMONIA			Ni- trates	Ni- trites				Lactose Gelatin at 20°			Lactose Agar at 37°		Anaerobic Lactose Agar at 20°	
			Total	Solu- tion	Sus- pen- sion						Acid	Liquefiers	Total	Acid	Total		
December 22																	
	A. M.																
543	9:30-10:30	20.0	5.7	3.6	2.1	0	1.80	72	3.40	600	180,000	—	230,000	380,000	380,000	420,000	
544	10:30-11:30	17.5	7.0	3.6	3.4	0	0.40	66	3.50	860	160,000	10,000	410,000	240,000	300,000	430,000	
545	11:30-12:30	18.5	5.5	3.5	2.0	0	0.40	49	3.30	1,200	200,000	10,000	280,000	300,000	340,000	410,000	
	P. M.																
546	12:30-1:30	15.0	5.5	3.4	2.1	0	1.80	46	3.50	2,500	280,000	5,000	380,000	830,000	840,000	200,000	
547	1:30-2:30	17.5	5.8	2.9	2.9	0	0.60	61	3.00	2,800	400,000	5,000	420,000	370,000	500,000	360,000	
548	2:30-3:30	14.5	6.2	3.0	3.2	0	1.00	62	3.50	3,200	400,000	10,000	420,000	760,000	790,000	360,000	
549	3:30-4:30	18.0	6.1	3.7	2.4	0	0.20	41	4.00	2,700	350,000	15,000	430,000	430,000	540,000	220,000	
550	5:00-6:00	18.0	6.3	2.9	2.4	0	0.20	53	3.30	2,200	310,000	—	410,000	320,000	430,000	230,000	
551	6:00-7:00	13.0	6.9	4.0	2.9	0.05	0.20	63	14.70	1,780	180,000	—	210,000	280,000	310,000	370,000	
552	7:00-8:00	10.0	4.9	2.9	2.0	0.05	0.20	57	8.40	240	130,000	—	140,000	70,000	100,000	320,000	
553	8:00-9:00	8.5	4.7	2.2	2.5	0.10	0.20	50	9.70	190	190,000	5,000	190,000	50,000	60,000	370,000	
554	9:00-10:00	10.0	5.5	2.3	2.2	0	0.10	50	12.10	480	280,000	10,000	280,000	210,000	250,000	400,000	
555	10:00-11:00	14.0	4.5	2.4	2.1	0	0.10	39	10.30	600	200,000	—	200,000	100,000	120,000	330,000	
556	11:00-12:00	10.0	4.7	2.6	2.1	0.05	1.00	—	6.50	710	100,000	—	110,000	110,000	130,000	520,000	
	December 23																
	A. M.																
557	12:00-1:00	11.5	3.5	1.7	1.8	0.20	0.10	28	12.00	680	40,000	—	50,000	5,000	50,000	410,000	
558	1:00-2:00	10.0	2.2	1.6	0.6	0.65	0.10	22	10.60	640	50,000	—	70,000	100,000	100,000	100,000	
559	2:00-3:00	7.5	2.1	1.0	1.1	0	0.10	21	7.40	670	100,000	5,000	100,000	70,000	80,000	270,000	
560	3:00-4:00	6.5	1.5	0.9	0.6	0	0.20	19	16.50	760	20,000	—	30,000	70,000	70,000	220,000	
561	4:00-5:00	3.5	5.0	4.2	0.8	0	0.20	21	15.10	980	40,000	—	40,000	40,000	40,000	130,000	
562	5:00-6:00	7.0	2.0	0.8	1.2	0	0.30	14	7.80	900	10,000	—	10,000	50,000	50,000	210,000	
563	6:00-7:00	7.5	3.3	1.6	1.7	0	0.20	16	6.50	1,000	30,000	—	40,000	5,000	20,000	1,860,000	
564	7:00-8:00	10.1	4.2	2.9	1.3	0	0.20	28	6.00	280	160,000	—	160,000	—	10,000	5,550,000	
565	8:00-9:00	15.0	5.0	2.9	2.1	0	0.20	45	4.60	180	80,000	—	100,000	20,000	50,000	260,000	
Averages.....		12.3	4.7	2.6	2.1	0	0.43	42	7.68	1,136	169,000	8,000	205,000	218,000	242,000	606,000	
Weighted averages		12.2	4.6	2.6	2.0	0	0.41	41	7.72	1,115	167,000	7,100	200,000	210,000	234,000	625,000	

TABLE 9.
Parts per Million¹

No.	Time	NITROGEN AS					OXYGEN CONSUMED	OXYGEN DISSOLVED	CHLORINE	NUMBER OF BACTERIA IN ONE CUBIC CENTIMETER						
		Free Am- monia	Albuminoid Ammonia			Ni- trates				Lactose Gelatin at 20°				Lactose Agar at 37°		Anaerobic Lactose Agar at 20°
			Total	Solu- tion	Sus- pen- sion					Ni- trates	Acid	Liquefiers	Total	Acid	Total	
	February 16	17.5	8.0	3.5	4.5	0.02	61	4.80	1,500	55,000	0	60,000	100,000	180,000	475,000	
610	11:30 A. M.	15.5	6.9	3.0	3.9	0.02	25	4.90	1,900	5,000	0	45,000	55,000	90,000	365,000	
611	12:30 P. M.	17.5	6.6	3.4	3.2	—	52	3.60	2,200	10,000	10,000	135,000	55,000	110,000	430,000	
612	1:30	15.0	6.7	3.2	3.5	0.02	61	—	2,500	30,000	10,000	80,000	50,000	95,000	185,000	
613	2:30	17.0	7.7	3.8	3.9	0.02	57	5.00	2,400	40,000	5,000	185,000	130,000	200,000	495,000	
614	3:30	16.0	6.3	3.6	2.7	0.00	60	—	2,400	115,000	10,000	300,000	170,000	335,000	475,000	
615	4:30	20.0	5.0	3.6	1.4	0.00	46	4.70	1,100	110,000	5,000	400,000	185,000	310,000	305,000	
616	5:30	30.0	4.7	2.7	2.0	0.00	42	—	540	330,000	5,000	430,000	70,000	145,000	325,000	
617	6:30	17.5	4.7	3.0	1.7	0.00	48	3.00	440	590,000	45,000	820,000	100,000	155,000	650,000	
618	7:30	15.0	4.9	2.6	2.3	0.02	30	—	400	340,000	35,000	1,400,000	185,000	200,000	400,000	
619	8:30	12.5	4.6	2.4	2.2	0.02	24	4.20	700	85,000	0	245,000	130,000	190,000	380,000	
620	9:30	10.0	3.1	1.6	1.5	—	27	—	600	315,000	10,000	420,000	100,000	175,000	180,000	
621	10:30	10.0	3.5	2.1	1.4	—	22	5.00	600	120,000	5,000	235,000	125,000	165,000	215,000	
	February 17	10.0	3.2	1.6	1.6	0.02	16	—	1,400	180,000	30,000	230,000	115,000	215,000	225,000	
623	12:30 A. M.	9.0	2.3	1.0	1.3	0.02	15	6.90	800	115,000	5,000	135,000	70,000	145,000	185,000	
624	1:30	6.5	2.2	1.1	1.1	0.01	11	—	700	60,000	15,000	180,000	20,000	65,000	145,000	
625	2:30	4.5	1.8	1.1	0.7	0.01	9	8.20	500	0	0	20,000	35,000	45,000	130,000	
626	3:30	4.5	1.8	0.9	0.9	0.01	10	9.00	600	20,000	5,000	60,000	15,000	25,000	150,000	
627	4:30	4.5	3.1	1.9	1.2	0.00	11	9.30	440	90,000	0	130,000	10,000	30,000	195,000	
628	5:30	4.5	2.7	2.0	0.7	0.04	28	—	300	65,000	5,000	170,000	35,000	65,000	100,000	
629	6:30	4.5	2.7	2.0	0.7	0.00	41	—	400	40,000	0	85,000	135,000	400,000	145,000	
630	7:30	10.0	8.0	6.0	2.0	0.00	46	—	280	50,000	10,000	115,000	95,000	135,000	115,000	
631	8:30	14.0	9.0	6.2	2.8	0.02	31	—	480	70,000	0	115,000	110,000	145,000	115,000	
632	9:30	11.5	5.2	3.0	2.2	0.04	74	—	300	110,000	25,000	200,000	145,000	180,000	280,000	
633	10:30															
	Averages	12.9	4.9	2.8	2.1	0.01	35	5.72	974	122,700	9,800	258,400	93,300	158,750	278,500	
	Weighted averages	12.8	4.9	2.8	2.1	0.01	35	5.73	951	122,000	8,900	261,000	93,500	156,000	294,000	

TABLE 10.

Parts per Million

No.	TIME	NITROGEN AS				OXYGEN CONSUMED	OXYGEN DISSOLVED	CHLORINE	NUMBER OF BACTERIA IN ONE CUBIC CENTIMETER							
		Free Am- monia	Albuminoid Ammonia						Ni- trates	Lactose Gelatin at 20°				Lactose Agar at 37°		Anaerobic Lactose Agar at 20°
			Total	Solu- tion	Sus- pen- sion					Acid	Liquefiers	Total	Acid	Total		
673	April 12 12:30 P. M.	4.5	5.0	2.2	2.8	44	5.6	500	158,000	75,000	350,000	6,500,000	6,800,000	230,000		
674	1:30	5.0	4.2	2.6	1.6	34	4.8	400	—	—	—	6,000,000	6,300,000	400,000		
675	2:30	9.0	3.5	2.6	0.9	59	5.7	300	60,000	40,000	300,000	140,000	430,000	150,080		
676	3:30	8.5	8.0	3.4	4.6	53	3.9	300	—	—	—	830,000	1,000,000	230,000		
677	4:30	—	—	—	—	54	3.4	200	60,000	30,000	700,000	350,000	380,000	315,000		
678	5:30	9.0	4.7	2.8	1.9	51	4.4	—	160,000	40,000	415,000	270,000	300,000	280,000		
679	6:30	9.0	4.5	2.4	2.1	66	6.1	380	105,000	65,000	615,000	1,200,000	1,200,000	340,000		
680	7:30	5.0	3.7	2.3	1.4	40	6.4	620	200,000	30,000	630,000	150,000	420,000	380,000		
681	8:30	8.0	3.3	2.0	1.3	47	6.9	770	85,000	10,000	280,000	450,000	480,000	305,000		
682	9:30	9.0	2.7	2.1	0.6	42	9.1	1,310	65,000	0	380,000	300,000	330,300	420,000		
683	10:30	10.0	4.7	1.6	3.1	39	8.5	2,330	90,000	20,000	280,000	200,000	240,000	120,000		
684	11:30	7.5	2.5	1.6	0.9	28	4.9	1,490	30,000	0	150,000	160,000	180,000	190,000		
685	April 13 12:30 A. M.	7.5	2.2	1.4	0.8	24	16.1	1,600	50,000	0	200,000	210,000	240,000	160,000		
686	1:30	9.0	2.7	1.6	1.1	31	10.1	2,820	55,000	25,000	250,000	200,000	230,000	235,000		
687	2:30	10.0	2.7	1.4	1.3	34	11.3	2,310	85,000	20,000	230,000	380,000	410,000	315,000		
688	3:30	7.5	1.1	0.8	0.3	17	9.9	1,740	55,000	0	255,000	140,000	160,000	140,000		
689	4:30	10.0	1.5	1.0	0.5	23	2.3	1,280	30,000	5,000	150,000	35,000	55,000	230,000		
690	5:30	7.5	1.9	0.8	1.1	14	4.5	800	10,000	15,000	65,000	80,000	85,000	120,000		
691	6:30	7.5	1.4	1.3	0.1	23	4.2	500	5,000	25,000	100,000	90,000	110,000	110,000		
692	7:30	9.5	1.8	1.5	0.3	22	5.9	540	10,000	0	95,000	110,000	130,000	70,000		
693	8:30	5.0	2.5	1.6	0.9	27	3.7	920	35,000	0	55,000	120,000	120,000	175,000		
694	9:10	10.0	3.7	2.8	0.9	39	8.9	1,560	130,000	10,000	195,000	230,000	240,000	250,000		
695	10:30	13.0	3.5	2.2	1.3	31	8.8	2,100	0	0	55,000	90,000	90,000	30,000		
696	11:30	17.5	2.2	2.0	0.2	32	4.5	2,400	320,000	15,000	550,000	150,000	160,000	150,000		
697	12:30 P. M.	17.5	2.5	2.2	0.3	29	—	2,700	900,000	30,000	1,300,000	930,000	980,000	—		
698	1:30	15.4	4.0	2.4	1.6	31	5.3	2,200	750,000	150,000	2,000,000	580,000	680,000	—		
699	2:30	6.5	3.0	2.2	0.8	30	4.3	2,000	—	—	—	—	—	—		
700	3:30	15.0	2.7	2.0	0.7	32	3.0	1,000	650,000	5,000	1,080,000	550,000	590,000	—		
Average for 24 hrs.		8.6	3.2	1.9	1.3	36	6.6	1,205	85,600	28,300	283,600	799,800	837,500	231,300		
Average for 28 hrs.		9.0	3.2	2.0	1.2	36	5.3	1,320	219,000	32,600	497,000	741,600	806,400	231,300		
Weighted average, 28 hrs.		9.0	3.2	2.0	1.2	36	5.3	1,320	219,000	32,600	477,000	741,600	806,400	231,310		

February, are typical of winter weather. Considerable snow and rain fell during the November run. Finally the last series (in April), followed heavy rain and spring thaws.

The analyses recorded in detail in Tables 5-10 are plotted in graphic form in Diagrams I-VI. Inspection of the curves shows, first, the general relation of the day and night flow. The nitrogenous bodies, indicated by the ammonias, and the carbonaceous constituents as measured by the oxygen consumed, rise to a maximum between 11:30 A. M. and 2:30 P. M., and fall to a minimum between 3 and 6 A. M. The curves for the dissolved oxygen naturally exhibit a reciprocal relation the amount increasing regularly from early evening to 6 or 7 A. M. The curves are most even and show the widest diurnal variation in the winter runs when the surface water was largely excluded and in the August series during the dry summer period.

Bacteriological analyses of sewage have never been carried out with so much detail so far as we are aware, and they bring out several points of interest. First, the enormous diurnal variations in warm weather are strikingly manifest in the diagrams of the July and August tests. The maximum occurs not at 11:30 A. M. when the organic constituents are highest, but from four to six hours later, at 4:30 P. M. We are inclined to attribute this mainly to the fact that the large amount of organic matter which passes in the morning is from the adjacent regions of Boston proper, and is comparatively fresh and undecomposed, while that arriving in the afternoon comes from the upper part of the system, so that the bacteria present have had time to multiply. The fact that the other four curves indicate no such enormous increase shows the important effect of the seasonal variations of temperature upon the multiplication of bacteria. The diurnal variation is, however, clearly shown even in December and February, although the absolute numbers are so small that the differences are somewhat obscured by the scale of our diagrams.

The effects of dilution with rainwater are shown with great clearness by the curves for the April analyses. The actual amount of nitrogenous matter present was much lower than at any other time, while the values for oxygen consumed remained as

high as in August and February, and not far below the July and December figures. This is attributable to the fact that while street washings are poor in nitrogen as compared with domestic sewage, they contain considerable carbonaceous matter derived from horse droppings, etc. The normal diurnal variations are, of course, somewhat obscured in the presence of much surface water, and this phenomenon is also evident in the April analyses.

GENERAL DIURNAL VARIATIONS IN THE COMPOSITION OF BOSTON SEWAGE.

Fuller (1903) and others have emphasized the importance of the hourly variations in the composition of sewage, and Goodnough and Johnson (1899) have published numerous series of analyses which bring out with clearness the extent of the variations. The absurdity of calculating sewage purification on the basis of analyses made during parts of the day when the sewage has twice its normal strength must be obvious, and yet this solecism is still commonly committed. Not only the hourly analyses but the hourly variations in flow should be considered; and in each of our tables there is given first the crude average for each constituent, and second, the true average corrected by weighting each analysis according to the amount of sewage flowing. In our case the correct average differs but little from the crude average, since the Moon Island sewer is usually full and shows but little variation in flow. Under other conditions, however, this allowance for hourly variations in flow would become of great importance.

We have calculated for the principal features of the analyses in Tables 5-10 the ratios which each hourly figure bear to the general average for the day, and have averaged the results obtained for each hour in the six different tests. The final figures in Table 11 probably form a fairly correct measure of the hourly variations in Boston sewage, and bring out some points not made clear in the diagrams. The maximum of albuminoid ammonia and oxygen consumed is seen to fall between 10 A. M. and 2 P. M., while the free ammonia is highest between 1 P. M. and 8 P. M. The latter thus follows the bacteria which is natural, since in all water and sewage analyses free ammonia, the product of bacterial decom-

position, and the bacteria which form it, are found to vary together. The dissolved oxygen is highest at 4 to 5 A. M., when ammonia, oxygen consumed, and bacteria alike reach their minimum. For purposes of comparison we have calculated similar

TABLE 11.

RATIO OF ANALYSES FOR EACH HOUR TO THE AVERAGE ANALYSIS OF THE TWENTY-FOUR HOURS (AVERAGE OF SIX SERIES).

TIME	FREE AMMONIA	ALBUMINOID AMMONIA		OXYGEN CONC.	OXYGEN DISS.	CHLORINE	BACTERIA		
		Total	Susp.				Gela- tin, 20°	Agar, 37°	Ana- erobic
10 A. M.	1.29	1.33	1.35	1.31	1.23	1.15	.77	.89	.60
11 "	1.27	1.27	1.19	1.19	.84	1.12	.81	.98	1.13
12 Noon	1.25	1.24	1.21	.98	1.35	1.17	.85	.70	1.04
1 P. M.	1.35	1.25	1.21	1.20	.96	1.61	1.04	1.17	1.11
2 "	1.40	1.29	1.49	1.31	1.17	1.71	1.05	.81	1.22
3 "	1.21	1.22	1.29	1.21	.94	1.71	1.14	1.42	1.16
4 "	1.31	1.07	1.03	1.09	.64	1.66	1.49	1.80	1.31
5 "	1.16	1.02	1.11	1.00	.84	1.18	1.09	1.15	1.02
6 "	1.38	1.07	.97	1.12	.74	1.06	1.43	1.64	.82
7 "	1.14	1.04	.96	1.20	1.18	.71	1.30	1.35	1.16
8 "92	1.20	1.42	1.00	.90	.52	1.90	.97	.86
9 "81	1.02	1.05	1.09	1.21	.58	.91	1.11	.95
10 "91	.97	.93	.96	1.13	.95	1.08	.72	.64
11 "97	.80	.75	.96	.80	1.30	1.67	.77	.98
12 Midnight75	.95	.94	.90	.74	1.33	1.10	2.25	.93
1 A. M.76	.86	.82	.71	1.07	1.04	.55	1.22	.85
2 "80	.61	.53	.74	1.21	.78	.48	.38	.40
3 "68	.83	1.06	.72	1.11	.63	.40	.47	.49
4 "52	.46	.51	.69	1.64	.57	.73	.30	.57
5 "75	.64	.56	.66	1.42	.90	.57	.21	.55
6 "62	.76	.79	.82	1.13	.40	.65	.59	.64
7 "63	.77	.67	.71	1.11	.47	.77	.67	1.24
8 "87	1.06	.91	.92	1.15	.56	.61	.63	.73
9 "95	1.15	.94	.93	1.04	1.04	2.18	1.31	.59

ratios for the six 24-hour runs reported by Goodnough and Johnson (1899), as Fuller (1903) has done for one of them. The results are presented in Table 12. Representing only a single series, the variations are less even than in our own table; yet they suggest distinct differences between the sewages studied. Marlborough, Spencer, and Natick sewages appear to be most concentrated in the afternoon, which is probably due to the length of the sewers above the point at which samples were taken.

TABLE 12.

RATIO OF ANALYSES AT DIFFERENT HOURS TO THE AVERAGE ANALYSIS OF THE
TWENTY-FOUR HOURS, IN SIX MASSACHUSETTS COMMUNITIES.

Spencer—June, 1898.

Hour	Rate of Flow	Residue on Evaporation			Nitrogen as		Oxygen Con- sumed	Chlo- rine
	Gals. per 24 hrs.	Total	Dissolved	Suspended	Free Ammonia	Albuminoid Ammonia		
8-9 A.M..	1.13	1.18	1.02	1.68	2.25	1.94	1.44	.90
10-11 " ..	.98	1.77	1.30	3.20	2.20	1.58	1.58	1.35
12-1 P.M..	1.29	1.11	1.03	1.38	1.85	1.49	1.35	.96
2-3 " ..	1.20	1.47	1.27	2.07	1.76	1.49	1.90	1.06
4-5 " ..	1.18	1.16	1.54	0.00	1.76	1.17	1.24	2.33
6-7 " ..	1.06	.85	.94	.58	1.68	.66	.76	.96
8-9 " ..	.97	.97	.96	.98	1.63	1.12	.96	.88
10-11 " ..	.90	.83	.81	.89	.97	.66	.84	.65
12-1 A.M..	.90	.53	.65	.15	.53	.33	.27	.59
2-3 " ..	.80	.52	.68	.05	.21	.15	.21	.56
4-5 " ..	.76	.48	.63	.03	.12	.14	.16	.55
6-7 " ..	.97	.59	.73	.18	.87	.34	.39	.69

Brockton—Outlet of Main Sewer, June, 1898

8-9 A.M..35	.53	1.10	1.80	1.63	.24	.42
10-11 "	1.35	1.09	1.74	1.62	2.07	1.74	.82
12-1 P.M..	...	1.20	1.30	1.07	1.70	1.54	1.60	1.24
2-3 "	1.30	1.37	1.21	1.66	1.44	1.34	1.45
4-5 "	1.51	1.45	1.61	1.40	1.44	1.41	1.72
6-7 "	1.02	1.13	.86	1.16	1.06	1.34	.89
8-9 "91	1.06	.70	1.32	1.10	1.03	1.01
10-11 "	1.15	1.40	.79	1.04	1.04	1.17	1.96
12-1 A.M..	...	1.17	.92	1.52	.81	1.08	.90	.85
2-3 "	1.31	.68	2.25	.59	.48	.68	.64
4-5 "38	.57	.94	.37	.30	.32	.55
6-7 "33	.52	.49	.24	.16	.25	.45

Framingham—Main Sewer, June, 1898.

9-10 A.M..	...	1.32	1.29	1.40	1.87	1.77	1.69	1.25
11-12 "	1.03	1.07	.94	1.45	1.26	1.16	1.04
1-2 P.M..	...	1.63	1.30	2.50	1.34	1.49	1.59	1.00
3-4 "	1.31	1.20	1.60	1.14	1.70	.23	1.24
5-6 "	1.04	.97	1.23	1.45	1.13	1.12	.76
7-8 "	1.13	1.03	1.40	1.48	1.19	1.18	.86
9-10 "82	.66	1.23	.67	.98	.82	.63
11-12 "57	.69	.28	.78	.43	.49	.77
1-2 A.M..47	.63	.05	.25	.26	.20	.60
3-4 "50	.67	.08	1.34	.21	.19	.58
5-6 "97	1.22	.34	.23	.34	.31	1.58
7-8 "	1.20	1.30	.98	1.22	1.23	1.00	1.46

TABLE 12.—Continued.

Marlborough—Main Sewer, June, 1898.

HOUR	RATE OF FLOW	RESIDUE ON EVAPORATION			NITROGEN AS		OXYGEN CON-SUMED	CHLO-RINE
	Gals. per 24 hrs.	Total	Dissolved	Suspended	Free Ammonia	Albuminoid Ammonia		
9-10 A.M. . .	1.03	.76	.79	.68	1.08	.84	.58	.75
11-12 " . .	1.13	1.57	1.58	1.55	2.51	1.71	1.55	2.55
1- 2 P.M. . .	1.10	1.15	1.06	1.36	1.65	1.95	1.19	.96
3- 4 " . .	1.17	1.36	1.19	1.78	1.12	1.35	1.72	1.09
5- 6 " . .	1.27	1.46	1.14	2.30	1.19	1.69	1.96	.94
7- 8 " . .	1.05	.97	.99	.94	.93	1.01	1.26	.79
9-10 " . .	1.02	.99	1.09	.75	.86	.87	.91	1.28
11-12 " . .	.94	.86	.92	.72	.82	.75	.78	.82
1- 2 A.M. . .	.90	.70	.97	.48	.59	.48	.52	.67
3- 4 " . .	.80	.57	.74	.14	.26	.24	.29	.57
5- 6 " . .	.77	.54	.69	.16	.86	.10	.12	.52
7- 8 " . .	.85	.53	.73	.00	.76	.07	.12	.56

Mattick—Main Sewer, June, 1898.

9-10 A.M.97	1.02	.40	2.35	1.47	.64	1.26
11-12 "	1.26	1.20	1.97	1.69	1.45	1.25	1.73
1- 2 P.M.99	1.02	.77	1.47	1.62	1.41	.94
3- 4 "	1.10	1.02	2.00	.70	.68	1.68	.96
5- 6 "	1.22	1.02	3.27	1.10	1.24	1.23	1.10
7- 8 "	1.18	1.04	2.75	1.33	1.73	2.23	.91
9-10 "99	1.06	.19	.90	.70	1.18	1.14
11-12 "85	.93	.05	.57	1.05	.59	.89
1- 2 A.M.87	.94	.14	.57	.51	.43	.79
3- 4 "86	.94	.10	.33	.23	.30	.77
5- 6 "84	.90	.29	.43	.75	.36	.75
7- 8 "86	.94	.05	.78	.70	.89	.79

Gardner—July, 1898.

9-10 A.M. . .	1.24	1.43	1.26	1.80	1.50	1.80	1.58	.99
11-12 " . .	1.16	1.60	1.59	1.62	1.57	1.55	1.67	1.14
1- 2 P.M. . .	1.23	1.29	1.31	1.25	1.07	1.07	1.10	1.27
3- 4 " . .	1.16	1.42	1.14	2.04	1.13	1.41	1.41	1.20
5- 6 " . .	1.07	1.23	1.37	.93	1.16	.99	.92	2.17
7- 8 " . .	1.03	.97	1.05	.79	1.41	1.17	1.10	1.16
9-10 " . .	.92	.67	.76	.49	.84	.66	.85	.75
11-12 " . .	.85	.84	.76	1.01	.98	.87	.99	.77
1- 2 A.M. . .	.74	.35	.43	.16	.35	.31	.22	.53
3- 4 " . .	.70	.28	.39	.06	.06	.15	.16	.38
5- 6 " . .	.77	.32	.44	.08	.16	.14	.19	.38
7- 8 " . .	1.14	.69	.74	.59	1.01	.87	.82	.61

By comparison with the tables above, it appears that the diurnal variations in Boston sewage are less wide than those characteristic of smaller towns. This is natural, since the long sewers of the larger system must tend to equalize local variations.

The practical conclusion of this work is, of course, the derivation of a constant by which the true composition of the sewage for 24 hours may be deduced from analyses made at a given time,—in the case of our experiments during the late morning. Reference to Table 11 shows that the ammonia and oxygen consumed ratios for Boston in the morning hours from 10 A. M. till noon range not far from 1.25 while the bacterial ratios on gelatin and agar are approximately .8. In using our routine daily analyses made on samples collected at this time as a measure of the average composition of the sewage this factor may be used as a correction.

MONTHLY VARIATIONS IN THE COMPOSITION OF BOSTON SEWAGE.

Our data for the study of seasonal variations consist of a series of daily analyses made during the last six months of 1903 and during April, May, and June, 1904. The examinations were unfortunately interrupted from January to March 1904. Averages of the analyses by months are shown in Table 13 with the pumpage at Moon Island in billion gallons per month as a correction factor. It must be noted that this is the total flow from the whole South Metropolitan system, not merely that which passes Albany Street; but the relative proportion of the latter must by months be practically constant. The crude average is first shown in the table, and next the corrected average, obtained by multiplying the analyses for each month by the flow for that month and dividing the sum by the total flow.

The monthly variations are better seen by a comparison of the ratios as they are shown in Table 14. The seasonal differences are much less important than those which subsist between the night and day flows; yet there is a well marked minimum in May, followed by a steady rise, culminating in November or December. The strength of the sewage bears a general inverse relation to the amount flowing, although from October to December both flows and analyses were high.

TABLE 13.

BOSTON SEWAGE—MONTHLY AVERAGE OF DAILY SAMPLES.

DATE	FLOW, BILLION GALLONS.	NITROGEN AS						OXYGEN CONSUMED, TOTAL
		Albuminoid NH ₃			Free NH ₃	Ni- trites	Ni- trates	
		Total	Solu- tion	Sus- pension				
April, 1904	4.0	5.2	2.8	2.3	15.8	.09	.00	41
May, 1904	4.2	5.7	2.5	3.2	14.9	.14	.00	31
June, 1904	3.8	7.0	3.0	4.0	19.8	.22	.48	43
July, 1903	3.4	6.3	3.2	3.0	18.2	.12	.48	47
August, 1903	3.3	7.1	3.8	3.3	22.4	.12	.00	45
September, 1903	3.4	6.3	3.0	3.2	21.4	.18	.00	45
October, 1903	3.8	6.3	3.4	2.9	20.5	.80	...	51
November, 1903	3.8	6.1	3.0	3.2	22.3	.20	...	53
December, 1903	4.2	6.6	3.6	3.0	24.0	.42	...	50
Crude average ...	3.8	6.3	3.1	3.1	19.9	.26	.16	45
Average corrected for flow	6.2	3.1	3.1	19.9	.26	.15	45
Final ave. cor. for diurnal variation		5.0	2.5	2.5	15.9	.21	.12	36

In the last line of Table 13 we have applied the diurnal variation factor, dividing the corrected averages above by 1.25.

TABLE 14.

BOSTON SEWAGE—RATIO OF MONTHLY ANALYSES TO GENERAL AVERAGE.

MONTH	FLOW	ALBUMINOID AMMONIA			FREE AMMONIA	OXYGEN CONSUMED
		Total	Solution	Suspension		
April, 1904	105	83	90	74	79	91
May, 1904	115	91	80	103	74	69
June, 1904	100	115	96	128	100	95
July, 1903	89	99	104	97	91	104
August, 1903	87	113	120	107	112	100
September, 1903	89	99	97	104	107	100
October, 1903	100	100	108	93	103	113
November, 1903	100	97	95	101	112	118
December, 1903	115	105	116	95	120	111
Nine months	100	100	100	100	100	100

For comparison we have calculated the monthly ratios for four other cities and towns, which are given in Table 15. The original analyses for Andover and Lawrence were obtained from the report of the Massachusetts State Board of Health for 1901

(Clark, 1902); the Worcester figures were taken from the reports of the superintendent of sewers from 1900 to 1903 (average of the four years), and the Brockton analyses from the reports of the City Engineer for 1900 and 1901 (average for the two years). All show October and November to be much higher than the other months. September is also high except at Andover; January and February are well above the average at Worcester; and January, February, and March at Lawrence. The minimum in each case occurs in April or May. There is, then, a yearly cycle in the composition of sewage as definite, if not as wide in range, as the diurnal cycle.

TABLE 15.
RATIOS OF THE MONTHLY AVERAGES OF SEWAGE ANALYSES TO THE YEARLY AVERAGE, FOR
FOUR MASSACHUSETTS COMMUNITIES.

Andover—1901.

MONTH	FREE AMMONIA	ALBUMINOID AMMONIA			CHLORINE	OXYGEN CONSUMED	BAC- TERIA
		Total	Soluble	Suspended			
January80	.43	.65	.17	.97	.51	1.21
February...	.75	.98	.28	1.84	1.11	.72	.87
March.....	1.02	1.10	1.18	1.00	.98	1.47	1.47
April.....	.73	.81	1.16	.39	1.02	.98	.87
May.....	.80	1.17	.99	1.38	.87	1.16	1.26
June.....	.80	.83	1.15	.68	1.20	.77	.68
July.....	1.00	1.57	.87	2.40	1.02	1.51	.25
August....	.70	.40	.52	.25	.70	.50	.02
September.	1.00	.50	.78	.17	.95	.53	.31
October...	1.36	1.21	1.39	1.00	1.07	1.25	1.52
November.	1.57	1.74	.83	1.63	1.20	1.63	2.89
December.	1.18	1.17	1.26	1.06	.90	1.01	.68

Worcester—1899-1903.

January ...	1.01	1.06	1.18	1.00	.99	1.15
February..	1.05	1.06	1.27	.94	.92	1.14
March.....	.75	.84	.89	.82	.76	.86
April.....	.64	.66	.67	.65	.74	.82
May.....	.87	.81	.82	.84	.88	.85
June.....	.93	.91	.84	.91	.98	.88
July.....	.95	1.04	.95	1.10	1.17	.99
August....	.94	1.04	1.00	1.07	1.17	.99
September.	1.32	1.24	1.25	1.12	1.33	1.05
October...	1.14	1.19	1.05	1.28	1.14	1.14
November.	1.21	1.23	1.14	1.29	1.09	1.20
December.	.82	.94	.96	.93	.84	.91

TABLE 15—Continued.
Brookton—1900-1902.

MONTH	FREE AMMONIA	ALBUMINOID AMMONIA			CHLORINE	OXYGEN CONSUMED	BAC- TERIA
		Total	Soluble	Suspended			
January ...	1.60	.99	.95	1.02	.84	.83
February ..	.93	.89	1.03	.80	.78	.90
March81	.67	.94	.58	.67	.84
April89	.78	.84	.75	.77	.79
May85	.93	1.00	.90	.79	.87
June80	1.00	.83	1.05	1.06	1.99
July99	1.02	1.09	.91	1.47	1.16
August96	.96	.91	1.01	1.42	1.07
September.	1.30	1.38	1.18	1.53	1.43	1.27
October ...	1.21	1.33	1.11	1.48	1.08	1.25
November .	1.21	1.17	1.13	1.17	.89	1.13
December .	.94	.85	.92	.81	.86	.88

Lawrence—Sewage at Experiment Station—1901.

January ...	1.21	1.25	1.40	1.12	.79	1.20	.62
February ..	.99	1.06	1.12	1.02	.62	1.12	8.00
March80	1.12	1.10	1.14	.64	1.41	.52
April	1.02	.97	1.14	.80	.89	.95	.50
May81	.74	.73	.75	.97	.80	.30
June	1.03	.90	1.00	.80	1.28	.93	.48
July79	.76	.80	.73	1.36	.80	.23
August	1.14	.91	.87	.96	1.03	.83	.09
September.	1.06	.77	.66	.89	1.37	.80	.11
October ...	1.14	1.18	1.00	1.33	1.20	1.07	.14
November .	1.24	1.44	1.34	1.53	1.24	1.31	.17
December .	.75	.89	.91	.89	.62	.80	.14

THE AVERAGE COMPOSITION OF BOSTON SEWAGE.

In estimating the strength of a sewage to be purified, both hourly and monthly fluctuations must be taken into account. The error due to seasonal variations is ordinarily eliminated by a series of analyses of day samples covering the entire year; and a correction for diurnal variation may be made with sufficient accuracy by applying a factor obtained from a series of hourly examinations. In the case of Boston sewage we have seen that analyses made between 10 A. M. and 1 P. M. bear to the average for the day the relation of 1.25 to 1.00. In the last line of Table 13 the corrected average of our daily analyses has been divided by the factor 1.25; and we believe that this fairly represents the general composition of the sewage passing the station at Albany

Street. In our daily analyses the months of January, February, and March are omitted and the results are so far imperfect; but since in the results for other cities, summarized in Table 15, these three months taken together do not depart far from the yearly average, we are inclined to believe this error not a very serious one.

To check this general representative analysis we have compared it in Table 16 with the straight average of the results of the six 24-hour examinations; and the agreement is seen to be close enough for any practical purposes. The representative analysis must of course be nearer the truth than the average of the daily samples of day flow or the average of six days, necessarily subject to individual variation.

TABLE 16.
BOSTON SEWAGE—AVERAGE COMPOSITION OBTAINED BY VARIOUS METHODS.

	NITROGEN AS						OXY- GEN CON- SUMED
	Albuminoid NH ₃			Free NH ₃	Ni- trites	Ni- trates	
	Total	Diss.	Susp.				
A. Average of daily analyses.	6.2	3.1	3.1	19.9	.26	.15	45
B. Average of six 24-hour runs	4.8	2.6	2.2	12.5	.09	.68	42.9
Representative analysis cor- rected by factor for diurnal variation	5.0	2.5	2.5	15.9	36

Figures for nitrites and nitrates cannot, of course, be corrected by the factor 1.25, since their diurnal distribution is quite different. It will be noted that the nitrates were more than four times as high in the 24-hour runs as in the regular day analyses, while the nitrites were much lower. This is explained by the fact that nitrates are most abundant during the night hours, when the sewage contains free oxygen. During the day, when organic matter is present in large amount, the nitrates are reduced, appearing in part as nitrites.

For purposes of comparison we have prepared Tables 17-19, showing the composition of sewages in a number of English, American, and German cities. The analyses are of differing value, some representing only a few samples, some a long series, the

data upon which they are based being stated in footnotes. In the case of Lawrence, analyses are available for a considerable series of years, and in Table 20 we have cited the figures obtained at five-year intervals in order to illustrate the increase often noticeable in the strength of sewage from year to year.

TABLE 17.
COMPOSITION OF SEWAGES—ENGLISH CITIES.
Parts per Million.

CITY	SOLIDS		NITROGEN AS				OXYGEN CON- SUMED 4 HRS. AT 80° F.	CHLO- RINE
	Total	Soluble	Free NH ₃	Organic Nitrogen (Kjel- dahl)	Alb. Ammonia			
					Total	Soluble		
Huddersfield	1061.	715.	10.7	—	6.0	—	99.0	123.
Leeds	1680.	1334.	23.6	—	11.3	—	126.0	—
Leicester	1680.	1045.	—	14.2	—	107.0	—
London (North)	1353.	870.	39.0	—	4.3	—	80.0	132.
London (South) ...	1501.	1093.	34.5	—	4.3	—	67.0	267.
Manchester	1310.	940.	24.0	—	6.0	—	118.0	170.
Belfast	1848.	143.	21.3	15.3	8.9	—	90.1	679.
Sutton	1628.	804.	114.3	—	8.85	—	57.1	114.
Aylesbury	1191.	824.	68.4	—	7.8	—	57.4	123.
Blackburn	1076.	602.	28.2	—	3.4	—	35.8	81.
Exeter	544.	299.	37.7	—	2.1	—	20.2	50.
Saltley	2190.	1455.	41.6	39.1	15.6	—	134.3	125.
Rea	1886.	1340.	35.0	48.3	15.2	—	173.8	245.
Hockley	2155.	1451.	33.7	59.7	16.7	—	140.4	216.
Aston	1832.	948.	51.7	92.8	19.5	—	133.3	203.
Dublin	1523.	1190.	32.4	—	40.1	—	87.8	201.

Huddersfield.—CAMPBELL, K. F., 1900, 1902. Average of five 24-hour tests with mean analysis of sewage from Aug. 1898 to Feb. 1900. Sewers on the combined plan.

Leeds.—HARDING, 1902. Mean analyses from Nov. 1898 to June 1900. Sewers on combined plan.

Leicester.—MAWBEY, E. G., 1903. Average of analyses made from Sept. 1898 to Sept. 1899. Combined plan.

London—Northern Outfall.—DIBDIN, 1884. Average analyses for 1893. CRIMP, S., 1893. Average analyses for 1893. DIBDIN, 1893. Average analyses for 1894. CLOWES, 1902. Sept. 1898, April 1899, Nov. 1900 to Aug. 1901.

London—Southern Outfall.—DIBDIN, 1893. Average for years 1893 to 1894. CLOWES, 1902. Nov. 1899 to July 1900.

Manchester—MANCHESTER, CITY OF, 1901. Period from May 16, 1900, to Jan. 24, 1901. Four daily samples per week, made up of hourly samples, which were mixed in equal portions.

Belfast.—LETTA, 1903. Twelve analyses, Jan. to June 1902.

Sutton.—DIBDIN, 1902. Twenty-seven analyses, Feb. 1897 to Aug. 1898,

Aylesbury.—DIBDIN, 1902.—Fifteen analyses.

Blackburn.—DIBDIN, 1902. Seventeen analyses.

Exeter.—DIBDIN, 1903. Twelve analyses.

Saltley.—REA, HOCKLEY AND ASHTON, WATSON, 1903. One analysis each.

Dublin.—CAMERON, 1903.

TABLE 18.
COMPOSITION OF SEWAGE—CONTINENTAL CITIES.
Parts per Million.

CITY	SOLIDS		NITROGEN AS				OXY. CONSUMED		CHLORINE	
	Total	Soluble	Free NH ₃	Organic N		Alb. NH ₃		Total		Soluble
				Total	Soluble	Total	Soluble			
(a) Halle..	3388.	2794.	89.	97.	59.					715.
Breslau ..	1178.	773.	73.							182.
(b) Halle..	2458.	1633.		55.	21.					209.
(a) Berlin.		695.	60.						294.	164.
Hamburg	1133.	922.	35.			5.06		54.	290.	312.
Cristiania	1097.	639.	19.	31.				62.		167.
Paris.....	630.		16.							50.
Zürich ...	607.	490.	8.	33.	18.					22.
(b) Berlin.	2172.	1068.	*	*						264.
Frankfurt	2062.	898.	63.	54.	11.			126.	—	30.

* Nitrogen as Free NH₃ + Soluble Organic N (by Kjeldahl) = 106.8.

Halle (a).—3 analyses. KÖNIG, 1887. With water closets.

Halle (b).—5 analyses. KÖNIG, 1887. No water closets.

Breslau.—Average 72 analyses. KÖNIG, 1887.

Berlin (a).—Average 14 analyses. BRUCH, 1899.

(b).—30 analyses. KÖNIG, 1886.

Hamburg.—Average of analyses from Sept. 1896 to March 1899. DUNBAR, 1899. Oxygen consumed by Kubel method.

Cristiania, Norway, 1902. Average 41 analyses during 1900-1901.

Paris.—Single analysis, 1900. STEURNAGLE, 1900; HOLST, 1902.

Zürich.—4 analyses. KÖNIG, 1887. No water closets.

Frankfort, 1891. Eight analyses. LEPSIUS, 1891.

TABLE 19.
COMPOSITION OF SEWAGES—AMERICAN CITIES.
Parts per Million.

CITY	SOLIDS		NITROGEN AS			TOTAL OXYGEN CONSUMED	CHLO- RINE
	Total	Soluble	Free Am- monia	Alb. Ammonia			
				Total	Soluble		
Brockton, Mass.	482.	381.	2.9	5.36	3.43	80.7	83.0
Framingham, Mass.	1031.	364.	21.9	8.46	3.17	97.9	64.2
Gardner, Mass.	508.	240.	24.7	8.12	3.18	57.3	42.0
Leicester, Mass.	747.	602.	29.3	6.86	3.73	21.6	56.2
Marlborough, Mass.	520.	341.	33.3	6.68	3.19	58.8	60.7
Medfield, Mass.	825.	696.	9.6	7.40	5.93	67.3	87.8
Natick, Mass.	349.	297.	12.5	2.58	1.59	29.3	57.0
Spencer, Mass.	293.	168.	13.4	3.91	1.61	32.9	30.8
Newport, R. I.	—	—	17.2	8.14	—	59.9	164.7
Plainfield, N. J.	530.	374.	31.7	12.8	—	58.25	49.6
Worcester, Mass.	870.	553.	15.7	5.97	2.23	93.0	80.9
Berlin, Ont.	—	—	20.9	16.74	—	499.5	61.0

DATA FOR TABLE 19.

Brockton, Framingham, Gardner, Leicester, Marlborough, Medfield, Natick, Spencer.—

Average of 12 monthly analyses during 1899. MASS. STATE BOARD OF HEALTH, 1900.

Newport, R. I.—Average of daily analyses from June 2 to Oct. 13, 1894.

Plainfield, N. J.—By ROLPH.

Worcester, Mass.—Average of 48 monthly averages of weekly analyses of sterilized daily samples, during 1900-1903 inclusive. The total solids are for the year 1901 only.

EDDY, 1900-1903.

Berlin, Ont.—Average of daily analyses from June 26 to Sept. 4, 1902. AMYOT, 1902.

TABLE 20.

YEARLY AVERAGE ANALYSES OF SEWAGE AT LAWRENCE, MASS.

Parts per Million.

YEAR	FREE AMMONIA	ALBUMINOID AMMONIA		OXYGEN CONSUMED (Kubel, 2 Min.)	BACTERIA PER 1 C.C.
		Total	Soluble		
1888	15.5	6.8	1.6	—	1,000,000
1893	26.8	6.3	3.1	34.5	923,000
1898	29.9	5.8	2.9	32.2	1,862,000
1902	44.9	7.2	3.1	40.6	3,377,400

Compiled from the Annual Reports of the Massachusetts State Board of Health.

Table 19 shows that in American sewages the free ammonia ranges from 3 to 30 parts and averages somewhere near 15 parts; figures for albuminoid ammonia vary from 2 to 15 parts and average near 7; oxygen consumed analyses lie between 20 and 100 parts and average about 60. Judged by these standards, Boston sewage is seen to be relatively strong in free ammonia and weak in albuminoid ammonia and oxygen consumed. The European sewages appear to be several times as strong as the American sewages in all constituents.

In Table 21 we have calculated the total amount of the various constituents in Boston sewage, multiplying the average daily analyses in Table 13 by the flow obtained from the pump records at Dorchester. We have assumed that the flow at Albany Street is 55 per cent of the total pumpage at Dorchester for the reasons previously cited, and the first column of Table 21 thus represents the estimated flow past the experiment station. The other figures are obtained by multiplying the analytical data in Table 13 by the estimated flow. The totals for the nine months are corrected by dividing by the factor 1.25; and finally the results are expressed in grams per capita per day.

TABLE 21.
BOSTON SEWAGE.
Total Amount of Various Constituents.

DATE	FLOW BILLIONS OF LITERS	THOUSANDS OF KILOGRAMS				
		Nitrogen as				Oxygen Consumed Total
		Albuminoid Ammonia			Free NH ₃	
		Total	Soluble	Suspended		
April, 1904.....	8.3	43	23	20	130	340
May, 1904.....	8.8	50	22	28	130	270
June, 1904.....	7.9	55	24	31	160	340
July, 1903.....	7.1	44	23	21	130	330
August, 1903.....	6.9	49	26	23	150	310
September, 1903..	7.1	44	21	23	150	320
October, 1903.....	7.9	50	26	24	160	400
November, 1903...	7.9	48	23	25	180	420
December, 1903...	8.8	58	32	26	210	440
Total, Nine Months	70.7	441	220	221	1,400	3,170
Total corrected for Diurnal Variation		352	176	176	1,120	2,536
Grams per Capita Daily		3.7	1.8	1.8	12.	27.

The values obtained are higher than those given for any other American sewage with which we are acquainted. Fuller (1903) offers as approximate average amounts of the various constituents in grams per capita daily, 15 for oxygen consumed, 7 for free ammonia, and 2.5 for albuminoid ammonia. Our figures are respectively 27, 12, and 3.7, from 55 per cent to 70 per cent higher. The analysis of Boston sewage as we have seen corresponds well with that of other American cities; its higher per capita content is due to the fact that its per capita flow is higher. The total amount of sewage pumped at Dorchester from July, 1903, to June, 1904, inclusive, was 46,000,000,000 gallons, which, with a contributing population of 477,000, makes a daily per capita flow of 264 gallons. Even using the total population of the South Metropolitan District, 669,000, we get a daily per capita flow of 188. Fuller's figures were all much lower than this,

the per capita flow at Framingham being 73, at Gardner 100, at Marlborough 113, and Worcester 115. The per capita flow appears to increase with the size of the community, as is the case with the per capita consumption of water, and it is natural that a city like Boston, with a considerable transient population, should exhibit this phenomenon to a marked degree.

It is at any rate evident that in dealing with cities of the first rank our estimates of per capita yield of polluting material must be materially modified; and we gain a new idea of the vastness of a problem which involves the treatment of 1,400,000 kilograms of nitrogen in the form of free ammonia in one only of the principal sewers of a city in a single year.

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THE NUMBER OF BACTERIA IN SEWAGE AND SEW-
AGE EFFLUENTS DETERMINED BY PLATING
UPON DIFFERENT MEDIA AND BY A
NEW METHOD OF DIRECT.
MICROSCOPIC ENU-
MERATION.

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INTRODUCTION.

THE main object in sewage purification is the oxidation or removal of its organic constituents to such an extent that the effluent shall not be subject to putrefactive decomposition. The aim of the process is therefore chemical and its success is gauged by chemical methods. The bacteriology of sewage and sewage effluents is, however, of importance since a knowledge of the bacteria which are the chief agents in the destruction of organic matter must make it easier so to adjust conditions as to obtain the maximum possible results. The study of the nitrifying bacteria, for example, has already yielded valuable data as to the effect of various chemical and physical agents upon these organisms. The admirable papers by Boullanger and Massol (1903), and by Schultz-Schultzenstein (1903), the latter translated into English by Kimberly (1904), are models of thorough and accurate investigation. The third paper of recent date on the same subject, by Fremlin (1903), fails to carry the conviction that errors of manipulation have been successfully avoided.

The bacteria active in the newer processes of sewage purification, in the septic tank and in the contact and trickling filters, have as yet received almost no attention; and it is hoped that the work planned at the Sanitary Research Laboratory of the Massachusetts Institute of Technology may to some extent supply this deficiency during the next few years. Investigations should be

made which are at the same time quantitative and qualitative, and they ought ultimately to include the detailed study of all the forms isolated from a series of plate cultures. I have thought that the ground might be cleared to some extent by quantitative analyses alone, so carried out under different conditions as to measure roughly certain principal groups of bacteria. With this end in view the following preliminary studies were undertaken.

PREVIOUS QUANTITATIVE WORK ON SEWAGE BACTERIA.

Determinations of the total number of bacteria present in sewage were made nearly 20 years ago by Wahl (1886), who found from 1,686,000 to 5,248,000 bacteria per c.c. in the sewage of Essen. Miquel (1891), records 13,800,000 per c.c. in the sewage at Gennevilliers with an average of 7,475 in the effluent from the broad irrigation fields. E. Ray Lankester (1892), at Oxford, England, found an average of 3,170,000 bacteria in sewage and 40,000 per c.c. in the effluent from a sewage farm. The Franklands (1894), note the presence of 26,000,000 in the sewage outlet at Ballater in Scotland. Laws and Andrewes (1894), record from two to eleven million bacteria in various London sewages.

It is probable that certain of these early experimenters did not appreciate the necessity for planting samples promptly to forestall the enormous multiplication which takes place soon after collection. Fuller (1895), and Winslow and Belcher (1904), have shown that a tenfold increase may take place under such conditions in 24 hours. In recent years English investigators, as shown in Tables 3 and 4 have found from two to five million bacteria per c.c. In Germany, according to Bruch (1899), the sewage of Berlin showed, in one series of analyses, 1,600,000 bacteria per c.c. and in another from 600,000 to 3,800,000. Examinations at Charlottenburg quoted by Grünbaum (1900), showed 2,700,000 bacteria in crude sewage and 225,000 in contact effluents.

In America the reports of the Massachusetts State Board of Health show that the sewage of Lawrence taken from the Lawrence Street sewer contained on the average from 1894 to 1901, 2,800,000 bacteria per c.c. Each year the Lawrence Experiment Station furnishes an immense accumulation of valuable data published in less detail in the last two reports than formerly. The monthly analyses of Lawrence and Andover sewage are extracted from the Report for 1900 to form the first two columns of Table 1; and Table 2 is compiled from the same volume (Clark, 1901), to show the total number of bacteria present in effluents of various types.

The third column of Table 1 is from a report on the Worcester purification plant by Eddy (1902); the yearly averages reported by the same author are as follows: Sewage, 3,712,000; septic tank effluent, 2,539,000; sand filter effluent, 41,900.

Analyses of American sewage outside the Massachusetts Reports are, unfortunately, rare. At Plainfield, N. J., Le Clear (1902) records the following average numbers of bacteria per c.c.: Crude sewage, 1,321,000; septic tank effluent, 556,800; contact filter effluent, 171,500. At Ames, Iowa, Walker (1901) and Pammel (1902) have carried out somewhat exhaustive studies.

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Their monthly results form the fourth column of Table 1; the general averages for the year 1901 are as follows: Sewage, 1,248,256; septic tank effluent, 991,298; sand filter effluent, 14,750.

TABLE 1.
BACTERIA IN SEWAGE. MONTHLY AVERAGES. BACTERIA PER C.C.
(Clark, Eddy, Walker.)

Month	Lawrence 1900	Andover 1900	Worcester 1901	Ames 1901
January.....	2,860,000	3,494,000	5,237,000	550,879
February.....	1,520,000	4,475,000	7,667,000	1,993,766
March.....	1,814,000	2,260,000	5,559,000	469,600
April.....	2,320,000	9,963,000	6,400,000	775,090
May.....	2,334,000	5,150,000	2,457,000	652,150
June.....	2,530,000	4,890,000	3,614,000	828,333
July.....	3,150,000	4,235,000	3,987,000	826,000
August.....	2,645,000	3,153,000	2,531,000	1,194,000
September.....	6,485,000	1,100,000	3,390,000	940,000
October.....	3,178,000	4,253,000	1,968,000	4,230,000
November.....	1,710,000	3,850,000	2,937,000	1,547,000
December.....	4,060,000	4,277,000		3,825,000

TABLE 2.
AVERAGE BACTERIAL ANALYSES OF EFFLUENTS FROM VARIOUS TYPES OF
FILTERS AND SEPTIC TANKS DURING 1900, AT ANDOVER
AND LAWRENCE, MASS. BACTERIA PER C.C.

Effluent from Septic Tanks	Effluent from Contact Filters	Effluent from Trickling Filters	Effluent from Sand Filters
1,209,500	94,500	74,200	73,900
1,929,000	486,200	69,700	1,175
	552,100		10,072
	386,400		1,243
	291,800		152
	543,900		10,300
	630,000		6,500
			16,300
			25,600
			1,485
			24,200
			252
			151
			23,600
			16,700
			4,700

So far only the total number of bacteria present has been considered; but in the various reports published by the Royal Sewage Commission of Great Britain and by the London County Council analyses are presented which go into greater detail. Woodhead (Rideal, 1899) found in Exeter

sewage, from 3,000,000 to 5,000,000 non-liquefying and 500,000 liquefying bacteria on aerobic plates and in anaerobic cultures, 300,000 liquefiers and 700,000 non-liquefiers. Klein and Houston (1899) report an average of 3,600,000 bacteria in sewage, of which 480,000 belonged to the *B. coli* group, with 1,300 anaerobic spore formers. Clowes (1898) gives the following figures for London sewage (Table 3).

TABLE 3.
BACTERIA IN LONDON SEWAGE. NO. PER C.C.
(Clowes.)

Source of Sample	Date	Total No. of Bacteria (Gelatin 20')	<i>B. Coli</i> and Closely Allied Forms	No. of Spores	Liquefiers	Spores <i>B. subtilis</i> <i>B. mesentericus</i> <i>B. mycoides</i> <i>B. megatherium</i>	<i>B. Fluorescens</i> Liquefaciens
Barking	Feb. 23 to May 4, '98	4,399,047	70,000	660	357,500	20	25,000
Crossness	Feb. 23 to May 2, '98	3,528,669	112,500	852	404,000	20	10,000

Rideal (1901) quotes figures for several other cities. At Exeter the crude sewage contained three to five million bacteria with 150,000 to 200,000 liquefiers, the tank effluent, one million with 300,000 to 400,000 liquefiers, and the final filter effluent, 900,000 bacteria with 100,000 liquefiers. At Chorley the crude sewage contained four million bacteria, the tank effluent 400,000, and the filter effluent, 46,000. At Leeds the crude sewage contained 2,500,000 to 3,000,000 bacteria per c.c. More exhaustive studies on London sewage and contact filter effluents were published by Clowes and Houston (1899 and 1903) in the second and third reports to the London County Council; these are summarized in Table 4.

TABLE 4.
BACTERIA IN LONDON SEWAGES AND EFFLUENTS. NO. PER C.C.
(Clowes and Houston.)

SEWAGE.							
Source of Sample	Date	Total No. of Bacteria (Gelatin 20')	<i>B. Coli</i> and Closely Allied Forms	Anaerobic Spores (Agar 37')	Aerobic Spores	Liquefiers	Total No. of Bacteria (Agar 37')
Barking	Oct. 16, '99, to Jan. 17, '00.	7,096,666	770,000				
"	Mar. 16, '99.	10,000,000	600,000				
Crossness	May 11, '98, to Dec. 21, '98.	7,357,662	600,000				
"	Jan. 11 to Feb. 22, '99.			554	340	1,076,923	
"	Mar. 22 to Oct. 4, '99.	5,711,000	655,555				
"	Aug. 2 to Oct. 4, '99.	5,758,571					2,802,857

TABLE 4—Continued.
CONTACT BEDS.

Source of Sample	Date	Total No. Bacteria (Gelatin 20°)	B. Coli and Closely Allied Forms	Anaerobic Spores (Agar 37°)	Aerobic Spores	Liquefiers	Total No. of Bacteria (Agar 37°)
Barking	Oct. 16, '99, to Jan. 17, '00.						
Bed A							
Primary ..		2,180,000	500,000				
Bed B							
Primary ..		2,700,000	200,000				
Bed A							
Secondary		1,918,571	200,000				
Bed B							
Secondary		1,444,285	233,500				
Barking.....	Mar. 16, '99.						
Fine rag stone bed.....		4,000,000	500,000				
Fine coke bed.....		1,800,000	300,000				
Crossness	May 11, '99, to Dec. 20, '00.						
4 ft. coke bed.....		4,968,666	400,000		252	806,666	
6 ft. primary coke bed		6,787,500	600,000		256	837,500	
6 ft. secondary coke bed		4,300,000	100,000				
4 ft. coke bed.....	Jan. 11, '99, to Feb. 23, '99.				320	833,333	
6 ft. primary coke bed.....				342			
6 ft. secondary coke bed.....				354			
13 ft. coke bed.....	Mar. 22 to Oct. 4, '99.	5,364,000	411,111	207			
13 ft. coke bed.....	Aug. 2 to Oct. 4, '99.	4,662,857					2,802,857

The sewage at West Derby and Walton, examined by Boyce (1900), showed the following results: Walton sewage, 13,400,000 bacteria per c.c.; West Derby sewage, 10,380,000; West Derby contact filter effluent, 614,000 (of which 10,000 were of the B. coli group and 100 anaerobic spore formers); West Derby sand filter effluent, 17,900 (of which 50 belonged to the B. coli group). Boyce, MacConkey, Grünbaum and Hill (1902) report the following results from the same locality (Table 5).

TABLE 5.
BACTERIA IN EFFLUENTS AT WEST DERBY. NO. PER C.C.
(Boyce, MacConkey, Grünbaum, and Hill.)

Source of Sample	Total Bacteria	B. Coli Group	Anaerobic Spores
Contact Filter.....	838,000	9,800	10-100
Sand Filter 1.....	79,800	125	Less than 1
2.....	50,700	590	Less than 1
3.....	31,700	49	Less than 1
4.....	113,800	432	Less than 1

Lorrain Smith (1903) presented to the Royal Commission on Sewage Disposal analyses of sewage and effluents at Belfast which indicated considerably higher numbers than those elsewhere recorded. His chief results are averaged and brought together in Table 6.

TABLE 6.
BACTERIA IN BELFAST SEWAGE. NO. PER C.C.
(Smith.)

Source of Sample	Total No. Bacteria (Gelatin 20°)	Liquefiers	Anaerobes	Aerobic Spores	B. Coli
Crude sewage.....	15,300,000	1,510,000	4,000,000	86	300,000
Screened and sedimented sewage.....	47,280,000	2,860,000	19,000,000	143	400,000
Effluents from primary contact beds.....	35,660,000	1,700,000	12,700,000	76	400,000
Effluents from secondary contact beds.....	21,850,000	1,120,000	9,680,000	62	200,000

With regard to certain groups, of alleged significance in sanitary water analysis, further data are available. Klein and Houston (1898, 1899) report in crude sewage from 30 to 5,000 spore-bearing anaerobes per c.c., and 90,000 to 2,000,000 organisms of the *B. coli* group. According to Houston (1899, 1902) crude sewage contains over 10 million bacteria per c.c., as determined by plating on gelatin, between one and ten million on agar at 37°, 100,000 organisms of the *B. coli* group, at least 1,000 sewage streptococci, and 1,000 to 10,000 anaerobic spore-formers. A recent investigation by Belcher and the writer (Winslow and Belcher, 1904), indicated somewhat smaller numbers of all these groups in American sewage. Samples were taken from a small lateral of the Boston system receiving very fresh domestic sewage, and the results might thus be expected to differ from those obtained in the London experiments. Anaerobic spores were found present in numbers less than 1,000 per c.c.; the *B. coli* group amounted to 28,000 per c.c. In these experiments all the colonies found upon dilute plates were fished and worked out in sufficient detail to place them in certain general groups whose characteristics are given in the original paper. The distribution in fresh sewage is shown in Table 7; the authors found in sewage stored in a glass-stoppered bottle that the bacteria of all groups multiplied tenfold within 24 hours, and then began to decrease.*

TABLE 7.
BACTERIA IN FRESH BOSTON SEWAGE.
(Winslow and Belcher.)

BACTERIA PER C.C.										
On Gelatin at 20°			Lactose Agar at 37°				Anaerobic Agar			
1,240,000			151,000				140,000			
Group.....	II	IV	Va	VIII	X	XI	XII	XIII	XIV	XV
Type.....	Cocci	Chromogenes	<i>B. subtilis</i>	<i>B. liquidus</i>	<i>B. coli</i>	<i>B. typhi</i>	<i>B. can-dicans</i>	<i>B. aerogenes</i>	<i>B. ubiquitus</i>	<i>B. rhinoscleromatis</i>
No. per c.c.	372,000	128,000	74,500	30,000	28,000	60,000	44,000	30,000	162,000	154,000

*For assistance in the collection of the foregoing references the author is indebted to Mr. G. C. Bunker.

SOURCE OF SAMPLES EXAMINED.

The Sanitary Research Laboratory of the Massachusetts Institute of Technology is situated near the junction of Albany street and Massachusetts avenue on the south side of Boston and on the line of the nine-foot main trunk sewer of the Boston Main Drainage Works. This is the principal vein of the South Metropolitan system receiving the sewage of Boston proper, Roxbury, Brighton, Allston, Newton, Brookline, Watertown, and Waltham, and its contributing population is over 300,000. At the station some 10,000 gallons a day are pumped from this sewer into three supply tanks $6 \times 4 \times 3$ ft. deep, from which it flows by gravity to the various experimental tanks and filters. As a rule, samples of the sewage were taken as it flowed from these supply tanks. Further statistics, with detailed monthly and hourly analyses of the station sewage will be found in another communication (Winslow and Phelps, 1905).

The septic tanks used in these experiments were cypress tanks, $6 \times 4 \times 4$ ft. deep. Four of them (5, 6, 8, 10) are closed tanks, and were first put in operation in June, 1903. Tank 6 is filled with $1\frac{1}{2}$ -inch broken stone. Tanks 7 and 9 are open tanks started in February, 1904. The contents of Tanks 5, 6, and 7 are changed once in 12 hours, of Tanks 9 and 10 once in 24 hours, and of Tank 8 once in 48 hours.

The contact filters studied are tanks $4 \times 4 \times 6$ ft. deep, or 4 ft. deep in the case of Nos. 17 to 20. No. 11 is filled with 2-3 in. coke; No. 12 with $1\frac{1}{2}$ -in. crushed stone; Nos. 13 and 16 with $\frac{1}{2}$ -in. crushed stone; No. 14 at first with 1-in. stone, later with tile-bricks arranged in regular open tiers. All these are single-contact filters receiving crude sewage in doses ranging from one to two million gallons per acre per day. Tanks 19 and 20 are primary contact filters of $1\frac{1}{2}$ -in. stone, and 17 and 18 are secondary beds of $\frac{1}{2}$ -in. stone. Seventeen and 19 receive septic sewage, 16 and 18 raw sewage at rates of one to two million gallons per acre per day for each bed. All were put in operation in June, 1903.

The trickling filters of the station are tanks $4 \times 4 \times 6$ ft. deep filled with $1\frac{1}{2}$ -in. crushed stone, and dosed by tipping buckets at a rate of 1,500,000 to 3,000,000 gallons per acre per day. Tank 15 takes sewage which has been septicized for 12 hours; Tank 23, septicized for 48 hours; and Tank 22, raw sewage. No. 15 was put in operation in July, 1903, and Nos. 22 and 23 in February, 1904.

The sand filters used are tanks $6 \times 4 \times 3$ ft. deep filled with 2 ins. of sand of effective size, .17 mm., resting on 6 ins. of coarser material. Tank 1 received 100,000 gallons of raw sewage per acre per day from June 30 to December, 1903, 200,000 gallons per acre per day for the first six months of 1904, and 400,000 gallons since June 24, 1904. Tanks 24 and 25 have received 400,000 gallons of septic sewage per acre per day since February, 1904. The sewage applied to No. 24 has been septicized for 24, that applied to 25, for 48 hours.

The bacteriological analyses were carried out in two series, one extending from July to December, 1903, and including examinations made twice a month, and the second comprising weekly analyses made in July and August,

1904. These are designated in the appended table as Series A and Series B respectively. In the analyses of Series A, I was assisted by Professor S. C. Prescott and Mr. E. B. Phelps, to whom I desire to express my thanks.

THE DIRECT MICROSCOPIC ENUMERATION OF BACTERIA.

Realizing that the ordinary culture methods reveal only a fraction of the bacteria present, I attempted to control them by a direct examination of sewage and effluents under the microscope, drying a measured volume upon a cover-slip of known area, and counting representative fields with a Sedgwick-Rafter micrometer such as is used for enumerating the larger micro-organisms in the examination of drinking water. In my first experiments I attempted to stain the bacteria in the liquid in which they were suspended by adding a few drops of methylene blue or gentian-violet to a one-ounce sample bottle of sewage. Methylene blue was soon discarded, because bacteria grew in the stain itself, and later carbol-fuchsin was substituted for gentian-violet because it was found to give larger counts. Finally the process of staining in the bottle was entirely abandoned. Five hundredths c.c. of the fresh sample was placed on the cover-slip, dried in the air, fixed in the flame, and stained with carbol-fuchsin by heating till steam appeared. The latter process stained the cells much more definitely and sharply, and gave higher and more constant results than those obtained by staining in the bottle. It is of interest to note that on many of the slides prepared by the earlier method some of the bacteria showed faint but unmistakable flagella, stained by the carbol-fuchsin without any mordant; and it is possible that some process by which staining reagents are added directly to liquid cultures in which bacteria are present in their most active state might give better results than the somewhat severe preliminary drying and fixing treatment to which they are usually subjected before staining.

The method of direct enumeration as finally developed offered no serious technical difficulties, and furnished constant and comparable results. Its accuracy and the significance of its results have been investigated more fully by Willcomb and myself in another communication (Winslow and Willcomb, 1905). It will there be shown that there are three main factors which might tend to make the microscopic count larger than the plate count; the inclusion of several bacteria in a single colony, the presence in the sample of dead bacteria in a stainable condition, and the presence of organisms which do not grow on our nutrient media. We shall show that with pure cultures of ordinary metatrophic bacteria the plate counts and microscopic counts closely correspond even when the number of bacteria present are rapidly decreasing. One hundred million cells per c.c. have disappeared in four hours without leaving any trace of stainable bacteria. It

appears, therefore, that the presence of dead cells introduces no serious error in the microscopic count—that its excess is due mainly to the presence of organisms which fail to appear upon our plates—and that it, therefore, furnishes a more accurate measure of the total number of bacteria present than do our ordinary methods.

If these conclusions are justified the results shown in Tables 8 to 12 represent the first attempt to determine the actual extent of the bacterial flora of sewage. The crude sewage itself contained on an average 29 million bacteria per c.c.; the septic tank effluents, 30 million; the contact filter effluents, 24 million; the trickling filter effluents, 17 million; and the sand effluents, 650,000. The ratio of the total number as determined by direct microscopic enumeration to the count upon gelatin plates was nearly 20 in the case of sewage, about 40 for the septic tanks and contact and trickling filters, and 70 for the sand filter effluents. This result is suggestive in view of the important rôle played in purification processes by the nitrifying organisms which are known not to develop upon our gelatin plates.

THE BACTERIA IN RAW SEWAGE.

The media used for these analyses included lactose gelatin, lactose agar and Nährstoff agar. The first two were made up according to the standard methods of the American Public Health Association, two per cent of lactose being added before the final filtration and the reaction adjusted to -0.5 on Fuller's scale. The Nährstoff agar contained one per cent of agar and one per cent of Heyden's Nährstoff dissolved in water and filtered through cotton.

Anaërobic cultures were made first by the Wright method (Wright, 1901), later according to the admirable modification of Rickards (1904), by inverting a tube or Erlenmeyer flask containing the inoculated and solidified medium in a tumbler of pyrogalic acid and caustic solution. Cultures incubated at 37° were counted after 24 hours; gelatin plates and anaërobic cultures at 20° after 48 hours, and Nährstoff plates after seven days. Acid production was observed on plates to which litmus had been added in the usual manner, both agar and gelatin plates in Series A and gelatin plates in Series B.

The average results of the analyses of sewage are presented in Table 8 with the ratio which the count on each medium bears to the count on lactose gelatin at 20°.

TABLE 8.
BACTERIA IN BOSTON SEWAGE.
SERIES A.

NO. OF SAMPLES	BACTERIA PER C.C.					
	On Lactose Gelatin at 20°			On Lactose Agar at 37°		On Anaerobic Agar
	Liquefiers	Acid Formers	Total	Acid Formers	Total	
56	365,000	1,670,000	5,430,000	1,670,000	3,760,000	2,440,000
Ratio to Gelatin Count.....	7	31	100	31	69	45

SERIES B.

No. of Samples	Bacteria per c.c.						
	Microscopic Count	Lactose Gelatin at 20°			Lactose Agar at 37°	Nährstoff	Anaerobic Gelatin
		Liquefiers	Acid Formers	Total			
25	29,000,000	149,000	429,000	1,690,000	1,400,000	2,930,000	850,000
Ratio to Gelatin Count.	1700	9	25	100	83	170	50

The total number of bacteria on lactose gelatin was three times as great in Series A as in Series B; this is due to the fact that the first series included the autumn months in which the number of bacteria in sewage reaches a maximum. In Table 9, the monthly values obtained in the first set of experiments show clearly this autumnal maximum which is also manifest in the Lawrence and Ames results of Table 1.

The average number of bacteria present in Boston sewage appears then to vary between 500,000 and 10,000,000 according to the season. These figures are, however, too high since they were obtained from samples collected during the daytime when the numbers are of course much higher than at night. Phelps and the writer have elsewhere (Winslow and Phelps, 1905), published

a series of hourly analyses which show the diurnal variation in the number of bacteria present to be very great. The numbers in Table 8 may, however, fairly be compared with those from other places which have generally been obtained in the same way by examination of day samples. They show that Boston sewage has the bacteriological composition which appears to be common to most of the European and American cities which have been examined. There is much less variation indeed than might have been expected. Of the localities for which bacteriological sewage

TABLE 9.
BACTERIA IN BOSTON SEWAGE. BY MONTHS. NO. PER C.C.

MONTH (1903)	ON LACTOSE GELATIN AT 20°			ON LACTOSE AGAR AT 37°		ON ANAEROBIC LACTOSE AGAR AT 20°
	Acid Formers	Liquefiers	Total	Acid Formers	Total	
July	445,900	314,300	2,995,000	420,000	1,864,300	3,480,000
August	318,750	150,000	4,263,600	1,133,000	2,688,900	1,461,500
September.....	4,021,900	850,000	11,487,500	3,268,750	8,504,400	4,557,500
October	635,000	95,700	3,693,000	1,298,300	1,407,000	639,300
November	530,400	15,000	587,100	530,600	551,300	605,100
December	655,000	8,700	712,000	762,000	814,000	696,000

analyses are quoted above, Essen, Berlin, Charlottenburg, Leeds, Exeter, Chorley, Oxford, Lawrence, Andover, Ames, Plainfield, Worcester, and Boston show results generally lying between 1 and 5 millions, London, Walton and W. Derby, figures varying from 2 to 10 millions, and Paris, Ballater and Belfast, over 10 millions. The accuracy of the latter results may be questioned since multiplication in storage so easily occurs.

In both series of analyses of Table 8 the ratios of the liquefiers and acid formers were remarkably constant. The liquefiers amounted to seven and nine per cent respectively of the total number of colonies on gelatin, a slightly lower figure than that obtained by English observers. Clowes (1898), found about 10 per cent of liquefiers; Woodhead at Exeter, about 10 per cent; Clowes and Houston (1903), 10 to 20 per cent; and Smith (1903), 1 to 15 per cent. The ratio of acid formers in Series A was 31 per cent both on gelatin at 20° and agar at 37°, and in Series B 25 per cent. It is significant to note the coincidence of these

figures which show that the acid forming organisms are in general so adapted to the body temperature that their counts at 37° are as large as at 20°, while the total number of bacteria on agar at 37° varies from 70 to 80 per cent of the total number on gelatin at 20°. Since in potable water the ratio of the 37 per cent count to the 20° count is generally under 10 per cent with acid formers absent, the application of the lactose agar plate to sanitary water analysis is apparent.

The anaërobic counts in Series A and B differ but little although made in one case on gelatin and in the other on agar. In each the anaërobic colonies were about half as numerous as the aërobic colonies on gelatin. In a few experiments with aërobic and anaërobic plates of Nährstoff the same general relation was found to hold.

Nährstoff agar incubated at 20° for seven days showed not quite twice as many bacteria as appeared on the gelatin plate in two days. This result is somewhat lower than that obtained by Gage and Phelps (1902), who report that with the count on Nährstoff agar as 100, that on gelatin was 34 after two days and 44 after three days when sewage was examined. With a sand effluent the gelatin count was less than 20 per cent and with river water, less than 10 per cent of the Nährstoff count.

BACTERIA IN SEPTIC TANK EFFLUENTS.

The analyses of the septic tanks studied, four in number in Series A, six in Series B, are summarized in Table 10; and for comparison the total numbers of bacteria on gelatin and the ratio to that number of the counts made in other ways are shown in Table 14 for all the various types of effluents examined.

The total number of bacteria shows a marked decrease after passing through the septic tanks, amounting to over 60 per cent in Series A and over 50 per cent in Series B. The Lawrence figures in Table 2 show a similar decrease compared with the sewage in Table 1; and at Worcester, Plainfield, and Ames the same phenomenon appears, the diminution amounting to about 50 per cent. At Exeter, Woodhead reported no such decrease. The ratios of the different groups to the total are almost identical with those obtained with crude sewage. Comparing the septic

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TABLE 10.
BACTERIA IN SEPTIC TANK EFFLUENTS.
SERIES A.

	NO. OF SAMPLES	BACTERIA PER C.C.					
		On Lactose Gelatin at 20°			On Lactose Agar at 37°		On Anaerobic Agar
		Lique-fiers	Acid Formers	Total	Acid Formers	Total	
Tank 5	15	1,650	291,000	665,000	298,000	454,000	700,900
Tank 6	14	180,000	525,000	1,660,000	550,000	1,300,000	810,000
Tank 8	15	202,000	360,000	1,244,000	484,000	730,000	900,000
Tank 10	12	290,000	880,000	3,830,000	1,210,000	1,840,000	1,360,000
Average	14	162,000	495,000	1,750,000	650,000	1,040,000	930,000
Ratio to Gelatin Count	9	28	100	35	59	53

SERIES B.

	NO. OF SAMPLES	BACTERIA PER C.C.						
		Microscopic Count	On Lactose Gelatin at 20°			Lactose Agar at 37°	Nährstoff	Anaerobic Gelatin
			Lique-fiers	Acid Formers	Total			
Tank 5 ..	8	21,525,000	78,000	291,700	926,700	682,500	1,399,400	610,000
Tank 6 ..	5	30,520,000	60,000	126,700	660,000	486,000	1,212,000	306,700
Tank 7 ..	5	42,140,000	37,500	210,000	708,000	456,000	1,271,000	230,000
Tank 8 ..	5	39,620,000	220,000	196,700	1,605,000	738,000	1,338,000	913,300
Tank 9 ..	4	24,425,000	33,300	80,000	34,300	322,500	958,750	163,300
Tank 10 ..	6	22,400,000	47,500	128,000	486,000	346,700	2,888,000	352,000
Average ..	5	30,105,000	79,400	172,200	787,800	505,300	1,511,190	429,200
Ratio to Gelatin Count	3,830	11	22	100	64	192	55

tank ratios with those of the sewage there is manifest in both series a relative decrease of the total on lactose agar at 37° and a very slight increase of anaerobes. The insignificance of the latter is somewhat surprising. A somewhat more marked increase is shown in the ratio of the Nährstoff count; and the ratio of the microscopic count to the gelatin count is doubled, the absolute value of the former being the same as in the case of the sewage.

These facts suggest that the decrease of 20° gelatin forms in passage through the septic tank may be balanced by a multiplication of other bacteria.

BACTERIA IN CONTACT FILTER EFFLUENTS.

The analyses of the contact filters, nine in Series A, and seven in Series B, are arranged in Table 11. They show in general that these effluents contained two-thirds as many bacteria as were found in the septic effluents and one-third to one-fifth as many as the crude sewage, or in absolute numbers 500,000 to 1,000,000. At Charlottenburg, Chorley, and Lawrence the numbers have varied from 100,000 to 600,000, while the effluents at Exeter showed one million and at London two to six millions. Smith at Belfast reports over 20 millions. In comparing the individual filters of Table 11 it may be noted that of the double contact beds, the primary pair, 19 and 20, show much higher numbers than the corresponding secondary beds, 17 and 18, 20, which takes septic sewage, being the highest of all.

The various groups of bacteria in contact effluents maintain the same relation as in the crude sewage, with two exceptions.

TABLE 11.
BACTERIA IN CONTACT FILTER EFFLUENTS.
SERIES A.

	NO. OF SAMPLES	BACTERIA PER C.C.					
		On Lactose Gelatin at 20°			On Lactose Agar at 37°		On Anaerobic Agar
		Liquefiers	Acid Formers	Total	Acid Formers	Total	
Tank 11	17	94,000	262,000	956,000	492,000	624,000	529,000
Tank 12	20	112,000	158,000	1,238,000	387,000	733,000	561,000
Tank 13	18	29,000	76,000	738,000	100,000	215,000	228,000
Tank 14	16	17,000	108,000	776,000	186,000	375,000	338,000
Tank 16	12	66,000	433,000	1,823,000	204,000	361,000	208,000
Tank 17	12	19,000	238,000	496,000	183,000	270,000	281,000
Tank 18	17	43,000	228,000	648,000	239,000	623,000	412,000
Tank 19	12	44,000	368,000	1,038,000	322,000	502,000	388,000
Tank 20	16	105,000	691,000	1,904,000	460,000	1,240,000	950,000
Average	15	60,000	270,000	1,060,000	290,000	570,000	440,000
Ratio to Gelatin Count	6	25	100	27	54	41

TABLE 11—Continued.

SERIES B.

	NO. OF SAMPLES	BACTERIA PER C.C.						
		Microscopic Count	On Lactose Gelatin at 20°			Lactose Agar at 37°	Nährstoff	Anaë-robic Gelatin
			Lique-fiers	Acid Formers	Total			
Tank 11..	5	22,600,000	94,200	215,000	689,600	455,000	939,700	510,000
Tank 12..	4	40,875,000	125,000	23,000	897,000	1,045,000	1,263,700	255,000
Tank 13..	5	21,260,000	47,500	93,300	287,500	190,000	1,324,000	250,000
Tank 14..	4	32,525,000	55,000	35,000	685,000	602,500	397,500	935,000
Tank 16..	4	15,827,500	135,000	36,700	240,000	186,700	450,000	257,500
Tank 17..	4	8,625,000	8,100	87,500	185,000	120,000	612,500	80,000
Tank 18..	4	27,225,000	135,000	200,000	682,500	442,500	1,265,000	427,500
Average .	4	24,133,900	85,700	126,800	520,900	434,500	891,800	387,900
Ratio to Gelatin Count	4,610	16	24	100	83	171	74

The proportion of liquefiers was increased in Series B to 16 per cent of the total on gelatin. In Series A this did not occur, possibly because the beds had not been in operation long enough to exhibit their typical characteristics; but in connection with the results obtained with the trickling filters the figures of Series B suggest that in a loose stone filter there may be an increase in the proportion of liquefying bacteria present. Clowes and Houston (1903), obtained negative results in this regard.

In the second place the ratio of bacteria as determined by the microscopic method showed a very great increase, being nearly fiftyfold that for gelatin; its absolute value was 24,000,000 against 29,000,000 for sewage.

BACTERIA IN TRICKLING FILTER EFFLUENTS.

The single trickling filter examined in Series A (Table 12), was not in thoroughly satisfactory operation and the analyses of its effluent are somewhat aberrant. Both series show a somewhat smaller total of bacteria than in the contact effluents, about one-quarter of the number present in raw sewage. The ratios of Series B, typical of the beds when in good working order, correspond almost exactly with those of the contact beds, except that they show a higher ratio of liquefiers, 30 per cent of

the total and nearly three times as many bacteria on Nährstoff as on gelatin. Apparently in the filters of these two latter types there is either a multiplication or a relative persistence of certain bacteria which do not grow on gelatin, and of the liquefying forms. Since nitrification and the dissolution of solid materials are among the most important functions of contact and trickling beds these phenomena are significant.

TABLE 12.
BACTERIA IN TRICKLING FILTER EFFLUENTS.
SERIES A.

	NO. OF SAMPLES	BACTERIA PER C.C.					
		On Lactose Gelatin at 20°			On Lactose Agar at 37°		On Anaerobic Agar
		Liquefiers	Acid Formers	Total	Acid Formers	Total	
Tank 15....	10	22,000	93,000	1,030,000	280,000	1,010,000	370,000
Ratio to Gelatin Count....		2	7	100	24	94	34

SERIES B.

	NO. OF SAMPLES	BACTERIA PER C.C.						
		Microscopic Count	On Lactose Gelatin at 20°			Lactose Agar at 37°	Nährstoff	Anaerobic Gelatin
			Liquefiers	Acid Formers	Total			
Tank 15....	8	23,633,000	127,500	98,800	258,700	410,000	1,228,500	228,700
" 22....	5	20,080,000	192,000	120,000	678,000	876,000	1,739,000	370,000
" 23....	5	9,425,000	20,600	124,200	415,000	66,500	557,000	240,000
Average....		17,729,000	133,700	114,100	450,600	284,200	1,174,800	279,600
Ratio to Gelatin.....		3,940	30	25	100	63	280	62

BACTERIA IN SAND FILTER EFFLUENTS.

The bacterial analyses of sand filter effluents in Table 13 correspond with those obtained at Chorley, Oxford, Lawrence, Worcester, and Ames in showing numbers varying from 1,000 to 50,000 per c.c. The West Derby and Exeter figures are somewhat higher.

TABLE 13.
BACTERIA IN SAND FILTER EFFLUENTS.
SERIES A.

	NO. SAMPLES	BACTERIA PER C.C.					
		On Lactose Gelatin at 20°			On Lactose Agar at 37°		On Anaerobic Agar
		Liquefiers	Acid Formers	Total	Acid Formers	Total	
Tank 1.....	14	120	470	1,220	330	630	320
Ratio to Gelatin Count.....		10	38	100	26	50	26

SERIES B.

	NO. SAMPLES	BACTERIA PER C.C.						
		Microscopic Count	On Lactose Gelatin at 20°			Lactose Agar at 37°	Nährstoff	Anaerobic Gelatin
			Liquefiers	Acid Formers	Total			
Tank 1.....	5	1,320,000	155	400	7,900	17,000	66,000	770
" 24.....	5	340,000	680	2,600	15,600	14,400	44,000	700
" 25.....	5	300,000	670	1,100	3,970	2,900	21,000	2,160
Average.....		650,000	500	1,380	9,160	11,400	43,600	1,210
Ratio to Gelatin Count.....		7,100	5	15	100	120	480	13

With regard to ratios on different media, three points are noticeable. The proportion of anaerobes in each series is distinctly lower than in any other type of effluent, being only half that of the sewage in Series A and less than a third in Series B. The ratio of the Nährstoff count and the microscopic count on the other hand is higher for the sand filter effluents than for any others, three times that of sewage for the Nährstoff count and four times that of sewage for the microscopic count.

CONCLUSIONS.

The results of this preliminary study may be briefly summarized as follows:

1. The day flow of Boston sewage contains on an average from one to five million bacteria per c.c. as determined by plating on

TABLE 14.

RATIOS OF COUNTS BY MICROSCOPIC METHOD AND BY PLATING ON VARIOUS MEDIA TO THE COUNT ON LACTOSE GELATIN AT 20°. SEWAGES AND EFFLUENTS.

SERIES A.

SOURCE OF SAMPLE	AVERAGE NO. OF BACTERIA PER C.C. LACTOSE GELATIN AT 20	RATIO OF NO. OF BACTERIA TO THE NO. ON LACTOSE GELATIN						AT 20°		
		Microscopic Count	On Lactose Gela- tin at 20°			On Lactose Agar at 37°		On Anaerobic Gelatin	On Anaerobic Agar	On Nährstoff
			Liquefiers	Acid Formers	Total	Acid Formers	Total			
Sewage.....	5,430,000		7	31	100	31	69		45	
Septic Tanks.....	1,750,000		9	28	100	35	59		53	
Contact Filters...	1,080,000		6	25	100	27	54		41	
Trickling Filters.	1,030,000		2	7	100	24	94		34	
Sand Filters.....	1,250		10	38	100	26	50		26	

SERIES B.

Sewage.....	1,680,000	1,700	9	25	100		83	50		170
Septic Tanks.....	787,000	3,830	11	22	100		64	55		192
Contact Filters...	520,900	4,610	16	24	100		83	74		171
Trickling Filters.	450,600	3,940	30	25	100		63	62		280
Sand Filters.....	9,160	7,100	5	15	100		120	13		480

lactose gelatin at 20°. Of these 7 to 9 per cent are liquefiers and 25 to 30 per cent acid formers.

2. Lactose agar counts at 37° show 70 to 80 per cent as many colonies as on gelatin at 20° with the same absolute number of acid formers as at the lower temperature.

3. Anaerobic cultures show about one-half as many colonies as corresponding cultures under aerobic conditions.

4. Nährstoff agar gives counts not quite twice as high as those obtained by the use of lactose gelatin.

5. Direct microscopic enumeration shows nearly 20 times as many bacteria as appear upon gelatin plates.

6. The bacteria in Boston sewage exhibit a marked seasonal variation with a maximum in September and a minimum during the winter months.

7. In passage through the septic tank the number of bacteria on gelatin falls off one-half, while 37° counts show a slightly less decrease. The microscopic count remains unchanged.

8. In passing through contact and trickling filters the number of bacteria on gelatin is reduced to one-third of its sewage value, or less. The proportion of liquefiers is doubled or trebled. The microscopic count is only from one- to two-fifths less than in the case of sewage.

9. Sand filter effluents contain about one-half of one per cent as many bacteria as raw sewage on the gelatin plate, over one per cent measured on Nährstoff agar and about two per cent as shown by the microscopic count. The anaërobes are more markedly decreased than the aërobes.

10. The count upon the gelatin plate, in sewage purification as elsewhere, appears to correspond well with the amount of decomposable organic matter. The number of bacteria as determined in this way, decreases with the amount of purification effected and may furnish an indirect measure of it.

11. The total number of bacteria as determined by direct microscopical enumeration does not decrease directly with the gelatin count. Its ratio to the latter is highest in the purest effluents. In view of the fact that the nitrifying bacteria do not appear on the gelatin plate this result is somewhat significant.

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THE MODE OF ACTION OF THE CONTACT FILTER IN SEWAGE PURIFICATION.

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INTRODUCTION.

THE contact system of sewage purification is now so well known that only a brief description of the process will be necessary in this paper. It consists in the use of a bed of coarse stone, coke, or other hard material, of from one-eighth inch to two inches in diameter; the bed is usually five or six feet deep, although greater depths have been used; is filled completely with sewage and allowed to stand full for a given period, usually two hours; it is then allowed to drain slowly and it stands empty for six or eight hours or longer.

This contact filter, or bacterial bed, as it was called at first, was introduced by Dibdin in the course of a series of experiments carried out by him for the London County Council on the sewage of London. The first of these experiments were made upon sand filters (intermittent downward filtration), and the contact filter was gradually evolved from this earlier type. The first contact filters were run continuously 16 hours out of 24, the outlet being so regulated that the filter-bed stood full during this period. It was then emptied and rested empty for a period of eight hours. Later these London beds were operated with a shorter full period as are all contact beds today.

THEORIES OF THE ACTION OF THE CONTACT BED.

Dibdin (1897),¹ in his earlier writings at least, seems to have been influenced in his conception of the action of the contact bed by the fact that, in his experiments, it was developed from the sand filter. He accordingly offers no special explanation of the manner in which the contact bed purifies sewage, assuming the action to be the same in the two types of filter. That such is the case we strongly doubt, since, it has never been shown that in a sand filter there is any extensive alternation of oxidizing and reducing reactions such as characterizes the working of the contact bed. In his testimony before the Royal Commission on Sewage Disposal, Dibdin (1902)² made the following statement concerning the action of the contact bed:

During that period of two hours rest, . . . each cavity becomes really a settling chamber. The solid matter in the sewage by the action of gravity, subsides or adheres to the larger particles as chips of wood in water will to a boat, and when you draw the water away from the bed these solid matters are left behind and the bacteria gradually extend over them, and in the course of time they become solutionized . . .

This view is certainly correct as far as it goes. It must be remembered, however, that half the organic matter of the sewage is already in a soluble state and will not be deposited in any such manner, and also that purification seems to be easier in the case of matter in solution than in that of suspended matter. That such is the case will be evident from the fact that purification goes on more rapidly in the case of clarified sewage.

Dunbar and Thumm (1902)³ carried out at the Hamburg Experiment Station an extensive study of the contact bed; and their conclusions in brief are as follows: The agencies of the contact bed are three in number: mechanical straining, physical absorption, and biological activity. They place the greatest emphasis upon the absorption phenomena which they demonstrate by well planned experiments but which they do not attempt to explain. Absorption, according to these investigators, is dependent upon (1) bacterial activity, and (2) aëration, and may be

¹ *The Purification of Sewage and Water*, London, 1897, p. 48.

² *Interim Report of the Royal Commission on Sewage Disposal*, London, 1902, 2, p. 232, Quest. 3905.

³ *Beitrag zum derzeitigen Stande der Abwasserreinigungsfrage, mit besonderer Berücksichtigung der biologischen Reinigungsverfahren*, Berlin, 1902.

checked by the removal of either of these factors. They are careful to state, however, that the action of the bacteria is simply to remove the absorbed material and thus prevent a saturation of the absorbing agent. The necessity of aëration is a similar phenomenon, the oxygen being necessary for the life of the bacteria in question. By the term "oxidation process" (*oxydationsverfahren*) which they apply to the contact system, it is not intended to indicate that the products of oxidation, such as nitrates, must be present in the effluent, but rather that oxygen is applied to the decomposable material at such an early stage and in such an amount that offensive putrefaction is prevented. The formation of nitrates and nitrites is, in the opinion of these authors, of secondary importance. Nitrates are formed only in the empty period and, in the author's belief, not exclusively by the Winogradsky organisms. Nitrites, in general, result from the reduction of the nitrates during the full period. No attempt is made to follow the course of these important compounds further.

Harry W. Clark (1902),¹ chemist of the Massachusetts State Board of Health, as a result of experiments carried out at the Lawrence Experiment Station, agrees with Dunbar that nitrification occurs more actively when the filter is standing empty, but he also adds that "nitrification certainly occurs when a filter is filled with sewage until such a time as the air is exhausted," and cites an experiment in which higher nitrates were found in the effluent at the end of the discharge than at its beginning. This must mean not only that the formation of nitrates is taking place during the full period but that it is taking place at a more rapid rate than is their reduction, there being an actual increase in the nitrates in the sewage. Clark further states (1903),² "that it has not been the general experience at Lawrence that good purification can be obtained without nitrification," and believes that nitrates are essential in a satisfactory effluent. His views are thus in so far opposed to those of Dunbar and Thumm.

The points at issue are not immaterial as might at first seem to be the case, but are of fundamental importance to the proper control of the process. Our chemical data show us the initial and

¹ *Annual Report of the Massachusetts State Board of Health for 1901, 1902*, p. 276.

² *Annual Report of the Massachusetts State Board of Health for 1902, 1903*, pp. 198, 203.

final states of the sewage as it enters and leaves the filter, but we have hitherto been in the dark as to the nature of the process itself. The purification of sewage has been likened to a combustion or burning up of organic matter and, judged by the end products alone, the reactions are not dissimilar. We know, however, that the process of oxidation which goes on in a filter is a much more complex phenomenon than any direct combustion. Many intermediate products are known in the former case which are unknown in the latter, and a closer study of the end products shows that they also differ considerably.

In the hope of throwing light upon the details and intermediate stages of this process and of determining the mode of action or, so to speak, the physiology of the contact bed, we have made an extensive series of chemical analyses of the contents of a working contact sewage filter, drawn from various levels of the filter and during various phases of its contact cycle with results which we believe to be of theoretical interest and practical importance.

TERMINOLOGY.

The term "bacterial bed" which was originally applied to the contact filter was inappropriate since all of the so-called biological filters are also bacterial filters. The term "contact filter" seems most fortunate emphasizing as it does the physical and chemical phenomena in the action of the filter.

The operation of the filter is divided into two periods; first, the period of filling and standing full, commonly and very naturally spoken of as the full period, and second, the period of emptying and standing "empty," often called the resting period. This term "resting period" seems to us to be peculiarly inappropriate, since as has previously been pointed out and as we shall have occasion to show in this paper, there is taking place at this time within the filter the most essential reaction of the whole process, namely, the oxidation of the nitrogenous and carbonaceous matter of the sewage. Moreover, while the term "full period" is not inappropriate, it at least is expressive only of the physical condition of the tank and gives no indication of the occurrence or nature of the important reactions which take place during that period.

In view of these facts we have deemed it advisable to apply to the various stages of the cycle a set of terms which shall be not only accurate as to fact but in so far as possible suggestive of the processes taking place during these stages. For the first stage, that of filling, we use the term "filling phase." The next period in which the filter stands full we have called the "reduction phase." The word reduction in this connection has a double significance, since as we propose to show, there are taking place during this period two strictly chemical reductions,—of the nitrates and of the nitrites,—and further, that in the broad sense the whole mass of sewage is being reduced, digested, or broken down into simpler forms both through the agency of the bacteria and through strictly chemical reactions.

For the third period of the cycle we prefer the term "emptying phase" to the more commonly used "draining period." Draining suggests the slow running off of the last portions of the sewage rather than the more rapid discharge of the whole dose and if used at all would be more appropriately applied to the latter stages of the emptying. Finally, for the last or empty period there seems to be no more appropriate term than "oxidation phase." This stage is the oxidation period *par excellence*, although there is also a certain amount of oxidation going on during the full or reduction phase. These terms, having been of service to us in our work, are therefore recommended for general adoption in the interest of both accuracy and uniformity.

PLAN OF THE WORK.

The study naturally divides itself into two parts corresponding to the two general periods in the operation of the filter. First, the filling and reduction phases, as already defined, and second, the emptying and oxidation phases. The study of the latter phase will naturally consist in analyses of the gas contained in the tank and of certain changes which take place on the filtering material, while in the former case it will be necessary to study the changes taking place in the sewage itself. In carrying out the work we have made use of the "oxygen cycle," instead of the usual "nitrogen cycle," attempting to follow the history of the

oxygen in the process and in this way to trace out the reactions of the successive phases.

Our experiments were carried out at the Sewage Experiment Station with filter Number 16, which is shown in the accompanying plate. This filter consists of a cypress tank, four feet square in area and six feet deep, filled with crushed trap-rock and granite of such a size that it would pass through a one-half inch mesh and be retained on a one-fourth inch mesh. The tank is provided with five pet-cocks, one foot apart, the first one being one foot below the surface of the stone. The pet-cocks, together with the effluent cock, provide means for sampling at six levels either the contained sewage during the reduction phase or the gases during the oxidation phase. The tank was first put in operation in June, 1903, and has been receiving three fillings daily of raw sewage at eight hour intervals for six days in the week. The method of operation has been to fill the tank in one hour, allow it to stand full for two hours, empty in one hour and allow a period of four hours before the next filling. The filter was out of service for one month in February, 1904. The averages of 78 analyses of the raw sewage and of 16 analyses of the effluent of this filter is given below. These analyses were made during the six months previous to the commencement of this work.

PARTS PER MILLION.

SAMPLE	TEMP.	NITROGEN AS				OXYGEN CONSUMED	OXYGEN DIS- SOLVED
		Free Ammonia	Albumi- noid Ammonia	Nitrites	Nitrates		
Sewage	62.0	21.3	6.89	0.30	0.08	50.0	3.7
Effluent	61.2	14.4	2.32	0.99	0.00	17.4	0.7

METHODS OF ANALYSIS.

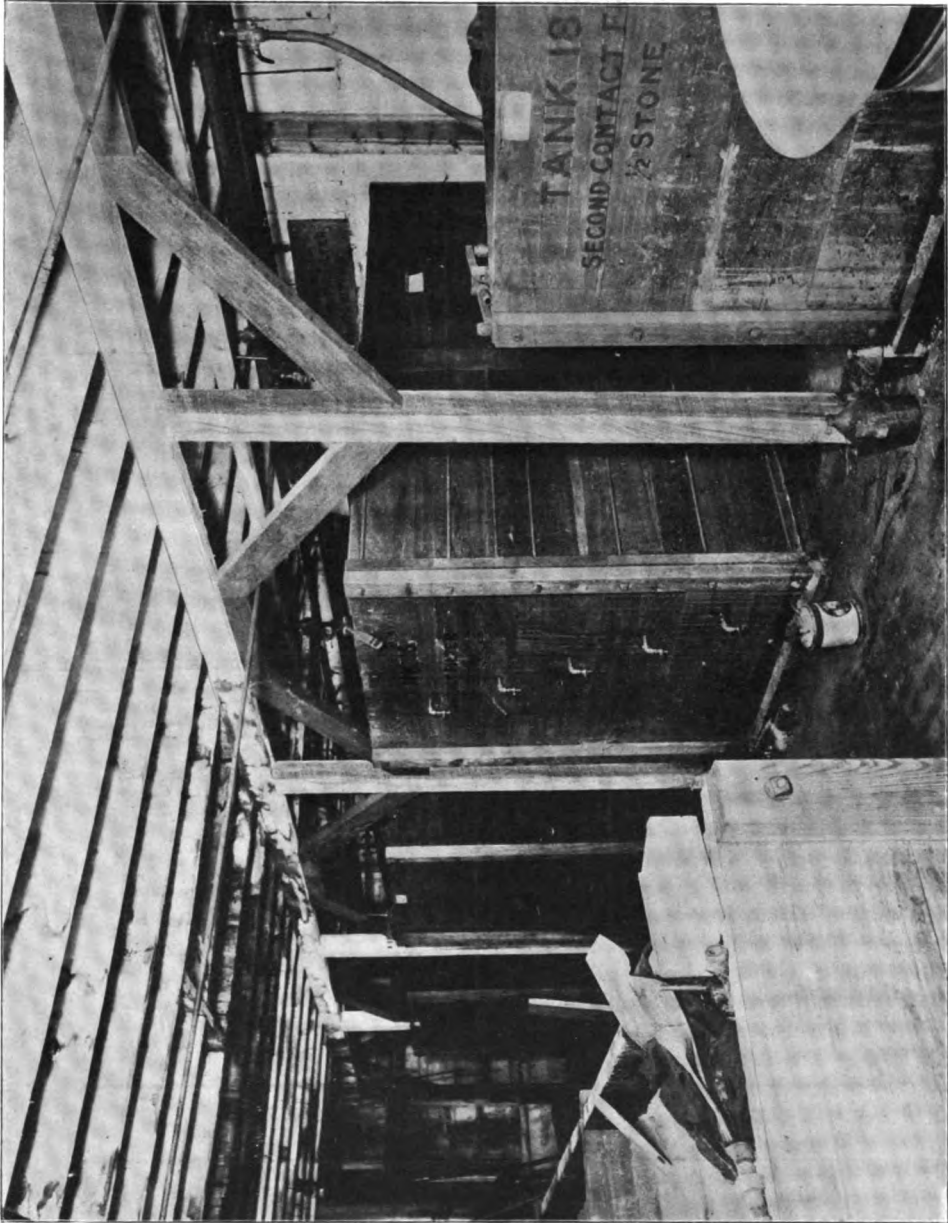
Analyses of the gas.—The gas analyses were made on a regular, portable, Orsat apparatus, as described by Gill (1897).¹*

Dissolved oxygen.—The determination of the dissolved oxygen was made according to the procedure of Winkler (1888).²

¹ *Gas and Fuel Analysis for Engineers*, New York, 1897, p. 11.

*The apparatus for this work was kindly loaned to us by Professor A. H. Gill, to whom the authors desire to express their thanks.

² *Berichte d. Deutsche Chem. Gesellsch.*, 1888, 21, p. 2843.



TANK 16 (Center Background)

Nitrates.—The determination of the nitrates was made by the Brucine method of Noll (1901),¹ as described by Farnsteiner and his associates (1902).²

Nitrites.—Nitrites were determined by the Griess method as described by Richards and Woodman (1904).³

Although the two general periods in the operation of the filter were studied successively throughout this work, it will aid in a clearer understanding of the results to present them separately.

OXIDATION PHASE.

Our work upon the oxidation phase consisted in studying the gas contained within the filter, the changes taking place in this gas during the phase and the difference in the composition of the gas at different depths within the filter. We also studied the chemical changes taking place upon the surface of the filtering material, and carried out some experiments with special apparatus

TABLE 1.

ANALYSES OF THE GAS FROM THE MIDDLE PET-COCK OF FILTER 16, TAKEN AT THE END OF THE FOUR HOUR OXIDATION PHASE.

DATE—1904	TEMP. C.	PERCENTAGES		
		Carbon Dioxide	Oxygen	Nitrogen (By difference)
March 10.....	47	0.4	18.5	81.1
“ 10.....	47	0.6	17.6	81.8
“ 16.....	49	0.4	18.8	80.9
“ 17.....	49	0.4	18.4	81.2
“ 18.....	48	0.4	18.8	80.8
“ 21.....	48	0.9	15.4	83.7
“ 23.....	48	1.3	16.4	81.9
“ 24.....	48	0.9	16.4	84.5
“ 26.....	53	0.8	17.7	81.5
“ 28.....	49	0.8	14.5	84.6
“ 29.....	50	1.5	16.7	81.8
April 2.....	49	0.8	16.0	83.2
“ 6.....	51	2.2	14.0	83.8
“ 8.....	51	1.5	17.5	81.0
May 9.....	56	2.2	17.5	80.3
Average.....	..	1.0	16.9	82.1
Air of the Filter House.	..	0.0	20.8	79.2

¹ *Ztschr. f. ang. Chemie*, 1901, 14, p. 1317.

² *Leitfaden für die Chemische Untersuchung von Abwasser*, Hamburg, 1902.

³ *Air, Water and Food*, New York, 1900, p. 94.

designed to test certain of the points brought out by our study. In Table 1 are given the analytical results of fifteen sets of gas analyses, together with an analysis of the normal air of the filter-house. The analyses were made at the conclusion of the four-hour oxidation phase and serve to illustrate the change taking place in the gas within the tank during that phase. The samples were in each case taken from the middle pet-cock.

A study of these figures shows clearly, first, that oxygen is absorbed or in some way used up in the filter and, second, that the whole of the oxygen thus taken up does not reappear as carbon dioxide, a portion of it disappearing entirely.

In order further to study the absorption of the oxygen by the filtering material we made the following experiments:

EXPERIMENT 1.

A bottle of about two liters capacity was filled with some material taken from the top of filter No. 16. This bottle was filled with sewage, allowed to stand for two hours, and then slowly emptied by means of a siphon. Air was immediately passed through it at a rate of about two liters per hour. Analyses of the air were made before and after passing through the bottle. The following results were obtained:

Sample	Carbon Dioxide	Oxygen	Nitrogen
Before passing	0.0	20.8	79.2
After passing	1.4	18.7	79.9

This experiment confirmed, as was anticipated, the results of Table 1, and shows that the absorption at the beginning of the oxidation phase takes place at a somewhat rapid rate.

EXPERIMENT 2.

To study those changes in the gases in a strictly quantitative manner, we devised a second experiment as follows:

The apparatus shown in Fig. 1 was used. *A* is a bottle filled with material taken from the top of filter No. 16. It was first filled with sewage and allowed to stand two hours; then the sewage was siphoned off and air admitted at *a*. The volume of the sewage removed, and thus of the air admitted, was measured. At the time of filling the bottle with sewage the whole apparatus was immersed in a pail of the same sewage in order to maintain as even a temperature as possible and especially to prevent sudden fluctuations.

The cylinder, *B*, was then placed in position and filled up to a definite mark with water, *a* being closed.

As oxygen was absorbed from the gas within the bottle, water was drawn in from *B*, and in order to make a reading at any time it was only necessary to add water to *B* from a graduate, filling it up to the original mark, and note the amount of water required. Temperature readings were made at the same time, and the proper corrections applied to the readings. Two series of observations were made with this apparatus, the results of which are given in Table 2. In each instance an analysis of the residual gas was made.

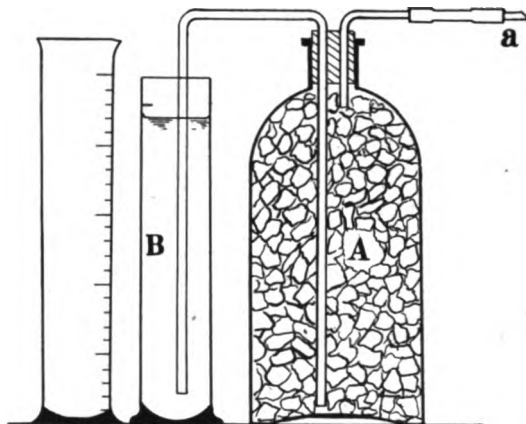


FIG. 1.

TABLE 2.
TABLE SHOWING APPROXIMATELY THE RATE OF ABSORPTION OF OXYGEN BY THE
FILTERING MATERIAL OF A CONTACT FILTER.

DATE	HOUR	TEMPERATURE	LOSS IN VOLUME		ANALYSIS OF RESIDUAL GAS	
			Measured	Corrected	CO ₂	O ₂
May 20.....	4 P. M.	15.0	Start		6.0	3.8
	10 P. M.	15.3	29 c.c.	30 c.c.		
21.....	4 A. M.	14.0	53 c.c.	49 c.c.		
	8 A. M.	14.0	64 c.c.	60 c.c.		
22.....	8 A. M.	17.0	93 c.c.	102 c.c.		
May 23.....	4 P. M.	13.0	Start		7.3	0.0
	5 P. M.	13.0	12.5 c.c.	12.5 c.c.		
24.....	11 A. M.	11.0	107.5 c.c.	100.5 c.c.		
25.....	11 A. M.	15.0	122.5 c.c.	129.0 c.c.		

Original volume in first series, 1,000 c.c., in second, 1,030 c.c.

Obviously if there are no other gases produced during the experiment, the total loss in volume will be the loss in oxygen minus the production of

carbon dioxide. Arranging our results from this point of view, we obtain the figures given in

TABLE 3.

	I	II
1. Original volume of air - - - - -	1,000 c.c.	1,030 c.c.
2. Loss in volume (observed) - - - - -	102 c.c.	129 c.c.
3. Per cent loss (observed) - - - - -	10.2%	12.5%
4. Oxygen absorbed - - - - -	17.0%	20.8%
5. Carbon dioxide evolved - - - - -	6.0%	7.3%
6. Loss in volume (calculated) - - - - -	11.0%	13.5%
7. Calculated loss (6) minus observed loss (3) - - -	0.9%	1.0%

It appears from these two sets of observations that about one-third of the total volume of oxygen used up within the filter during the oxidation phase reappears as carbon dioxide, and that the remainder is held on the surface of the filtering material in some fixed form, probably as nitrates. In addition to this loss of oxygen there is the production of an inert gas to the extent of about one per cent of the total volume. This gas is probably nitrogen, as we shall show in our discussion of the reduction phase.

A few sets of analyses were made at the beginning of the oxidation phase of the gases at the different levels, and the differences observed were either very slight or altogether wanting. There is practically no variation in the composition of the gas throughout the tank at this stage.

In order to study the chemical changes taking place on the surface of the filtering material, we made the following experiment:

We prepared six wooden boxes, four inches square and four inches deep inside, with perforated bottoms and open tops. These boxes were buried in the material of filter No. 16 and were then filled with the material displaced. The tops of the boxes were flush with the surface of the filtering material. They thus constituted portions of the tank itself which could be removed at any time for examination. Three days later, and immediately after drawing down the sewage at the end of one of the two-hour reduction phases, one of these boxes was removed and its contents used to fill a wire basket of about one liter capacity. This basket was then washed by pouring one liter of water through it four times, and the wash water thus obtained was examined for nitrates and nitrites. Other boxes were removed later, at intervals, and similarly examined. In this way it was possible to follow the nitrate and nitrite production in the filter during the oxidation phase without disturbing the main body of the filter. The results obtained in these experiments are given in Table 4.

TABLE 4.
FORMATION OF NITRATES AND NITRITES ON THE SURFACE OF THE
FILTERING MATERIAL OF A CONTACT FILTER DURING
THE OXIDATION PERIOD.

TIME	NITROGEN AS	
	Nitrates	Nitrites
11:30 A. M.	0.0003 mg.	0.0006 mg.
12:00 M.	0.0009 mg.	0.0001 mg.
I. 1:00 P. M.	0.003 mg.	0.0001 mg.
4:00 P. M.	0.004 mg.	0.0001 mg.
II. 4:00 P. M.	0.009 mg.	0.0000 mg.
III. 4:00 P. M.	0.007 mg.	0.0001 mg.

Outlet cock opened and tank started to empty in each case at 11:30.

The first four determinations constitute one series, and show in a general way the formation of nitrates and the loss of nitrites during the oxidation phase. The two single sets were made on two other days at the end of the four-hour oxidation phase.

The significance of the figures lies in their relation to the free oxygen in the bed. It was seen in Experiments 1 and 2, and in the analyses of the tank gases given in Table I, that a large amount of oxygen was used up in the bed during this phase which did not reappear as carbon dioxide. A portion of that oxygen is evidently used up in the production of nitrates.

In addition to these experiments some data on this same point may be gathered from the analyses of the sewage within the tank during the filling period. During a portion of the time the night filling of this tank was omitted and it had therefore a twelve hour oxidation phase alternating with one of four hours. On filling with sewage and examining the first sewage passing through the bed of the filter the nitrates were found to be much higher after the longer period of standing empty. For example:

Time	After Empty Period of	Nitrogen as Nitrate Parts per Million
April 22, 4 P. M.	4 hours	1.11
April 23, 8 A. M.	12 "	5.00

These figures also show that the production of nitrates goes on rapidly during the empty period, and that a longer period of rest gives a corresponding amount of oxidized nitrogen.

In general the reactions of the oxidation phase appear to be as follows. The organic matter within the filter-bed is oxidized at the expense of the free oxygen. A part of the carbon is converted into carbon dioxide and escapes in the gaseous form. Some of the nitrogen is converted into the higher oxides, nitrogen trioxide and nitrogen pentoxide. All these reactions taken together, however, do not account for more than two-tenths of the oxygen used up. A large portion of this gas must go toward the partial oxidation of the organic matter, producing compounds which contain sufficient oxygen to render them stable and unobjectionable in an effluent, but which are, nevertheless, capable of further oxidation. Such bodies we know do exist in perfectly satisfactory effluents, in which the "albuminoid ammonia," and "oxygen consumed" values may be quite high, but which, if submitted to the incubation test, are found to be non-putrescible.

THE REDUCTION PHASE.

Our studies of this phase consisted in analyses of the sewage as it flowed in, samples being taken from the various pet-cocks, and also of the sewage at various intervals during the period, to ascertain what changes were taking place within the filter. Determinations of the four conditions of the oxygen, namely the free and dissolved oxygen, and the oxygen as carbon dioxide, nitrates and nitrites were made. The analyses given in Table V, under the heading "initial," were obtained by allowing the sewage to flow into the tank as usual and, starting with the lower pet-cock, taking a sample of the sewage from each pet-cock, as it reached that point. In this way the samples represented successive washings of the stones above. The pet-cocks are lettered in color, A being at the top. At the end of the two-hour period similar samples were taken. The results are given in Table 5 under the heading "final."

This table shows in a general way that, in the action of the contact filter, the dissolved oxygen and the oxygen of the nitrates

TABLE 5.

ANALYSES OF THE APPLIED SEWAGE, AND OF THE SEWAGE WITHIN THE BED OF A CONTACT FILTER, TAKEN FROM FIVE LEVELS AT THE BEGINNING AND THE END OF THE REDUCTION PERIOD.

(Parts per Million.)

DATE TEMP. F.	PET-COCK	DISSOLVED OXYGEN		NITROGEN AS			
				NITRATES		NITRITES	
		Initial	Final	Initial	Final	Initial	Final
March 25 44°	Applied sewage	7.8	...	1.0	...	0.02
	A	7.5	0.0	0.9	5.6	0.05	0.12
	B	8.5	0.0	1.0	1.2	0.05	0.60
	C	8.9	0.5	0.9	1.7	0.06	0.80
	D	9.5	0.0	1.3	2.3	0.06	0.05
	E	9.7	0.0	3.0	1.7	0.06	0.09
	Average	8.7	0.1	1.4	2.5	0.05	0.33
March 28 44°	Applied sewage	7.8	...	0.30	0.06
	A	5.9	0.7	1.30	0.50	0.09	0.40
	B	8.3	1.4	1.80	1.10	0.09	0.70
	C	8.1	2.8	1.30	0.85	0.10	1.00
	D	6.8	0.9	1.30	1.00	0.06	0.58
	E	9.3	0.2	2.15	1.45	0.08	0.22
	Average	7.7	1.2	1.4	1.00	0.08	0.58
March 30 51°	Applied sewage	9.1	...	0.40	0.132
	A	5.5	1.9	1.35	0.90	0.444	0.500
	B	6.0	0.4	1.85	0.50	0.444	0.400
	C	6.4	0.2	0.85	0.00	0.284	0.150
	D	6.4	0.1	1.10	0.00	0.200	0.050
	E	7.5	1.1	2.25	0.40	0.136	0.065
	Average	6.8	0.7	1.30	0.36	0.273	0.233
March 31 51°	Applied sewage	9.4	...	0.30	0.160
	A	4.6	0.0	0.80	0.10	0.340	0.10
	B	5.5	0.5	0.60	0.15	0.300	0.40
	C	6.3	2.1	0.60	0.40	0.240	0.70
	D	5.5	1.6	0.65	0.40	0.260	0.65
	E	9.5	0.8	1.65	1.10	0.200	0.40
	Average	6.8	1.0	0.60	0.43	0.250	0.45
April 1 49°	Applied sewage	9.1	...	0.14	0.168
	A	5.0	0.0	0.20	0.03	0.276	0.16
	B	5.5	0.0	0.18	0.02	0.200	0.04
	C	5.9	0.0	0.16	0.02	0.200	0.00
	D	7.1	0.1	0.18	0.01	0.234	0.02
	E	7.7	0.4	0.40	0.22	0.200	0.20
	Average	6.7	0.1	0.21	0.06	0.213	0.08
April 12 51°	Applied sewage	4.4	...	0.13
	A	2.3	0.0	0.35	0.16
	B	3.8	0.2	0.34	0.02
	C	4.8	0.6	0.45	0.30
	D	5.0	0.4	0.80	0.60
	E	6.0	0.1	1.17	0.71
	Average	4.4	0.3	0.54	0.36

TABLE 5—Continued.
(Parts per Million.)

DATE TEMP. F.	PET-COCK	DISSOLVED OXYGEN		NITROGEN AS			
				NITRATES		NITRITES	
		Initial	Final	Initial	Final	Initial	Final
April 18 53°	Applied sewage	3.5	...	0.06	0.052
	A	2.3	0.0	0.32	0.00	0.104	0.000
	B	3.4	0.0	0.32	0.00	0.112	0.000
	C	3.2	0.2	0.70	0.02	0.108	0.400
	D	4.0	0.0	0.80	0.49	0.118	0.120
	E	5.8	0.0	2.30	1.00	0.108	0.000
	Average	3.7	0.0	0.90	0.30	0.100	0.104
April 20 52°	Applied sewage	5.6	...	0.10	0.064
	A	1.6	0.4	0.39	0.14	0.228	0.500
	B	4.7	...	0.39	0.03	0.258	0.200
	C	5.4	0.0	0.78	0.18	0.200	0.390
	D	6.7	0.0	1.00	0.59	0.140	0.195
	E	5.4	0.0	1.11	0.72	0.104	0.000
	Average	4.9	0.1	0.63	0.33	0.166	0.257
April 22 53°	Applied sewage	4.1	...	0.11	0.263
	A	1.1	0.0	1.72	0.10	0.370	0.252
	B	1.7	0.6	2.94	0.40	0.418	0.312
	C	2.2	0.3	3.34	1.32	0.434	0.498
	D	2.7	0.3	3.57	1.38	0.400	0.334
	E	3.1	0.4	5.00	1.79	0.400	0.338
	Average	2.5	0.3	2.78	1.00	0.381	0.347
Averages 51°	Applied sewage	6.76	0.282	0.115
	A	3.98	0.33	0.803	0.837	0.238	0.254
	B	5.22	0.40	1.047	0.380	0.234	0.332
	C	5.68	0.74	1.009	0.532	0.203	0.492
	D	5.96	0.38	1.190	0.751	0.184	0.250
	E	7.11	0.33	2.114	1.010	0.161	0.184
	General average	5.78	0.40	1.070	0.702	0.189	0.298

are used up during the reduction phase, the latter being reduced to about 70 per cent of its original value and the former to about seven per cent. The nitrites show an increase of about 50 per cent. A study of the initial values at the various levels, however, makes it appear probable that a much more complex reaction is taking place than would be indicated by a mere statement of the final results. It will be noticed that the initial samples from the bottom pet-cock *E* show in each case the largest amount of dissolved oxygen and also of nitrates. This is what might be expected since the latter are being washed from the filtering

material and the former is being absorbed from the air with which the tank is filled, as the sewage passes in thin films over the stones of the filter. One would also expect to find a similar relation in respect to the nitrite value. As a matter of fact, however, the largest amount of nitrite is found either at one of the middle pet-cocks or even, in one case, at the top. Since nitrites are very soluble the only explanation that we are able to offer of this important fact is that the nitrites are rapidly changed by reaction with the incoming sewage and, during the time that it takes the sewage to trickle over the stones to the lower levels, this reaction causes a very noticeable decrease in the amount of nitrites present.

It is of special importance to note the extreme reactivity of the nitrites in this process as compared with the much more stable behavior of the nitrates. In our conclusions we shall take up this reaction again and call attention to its great importance in the action of the filter.

TABLE 6.
ANALYSES OF THE SEWAGE FROM THE BOTTOM PET-COCKS AT
THE TIME OF FILLING AND 20-MINUTE
INTERVALS THEREAFTER.
(Parts per Million.)

TIME	DISSOLVED OXYGEN	NITROGEN AS	
		Nitrates	Nitrites
April 7,			
8:00 A. M.	3.13	1.28	0.74
8:20	1.66	0.77
8:40	2.73	2.00	1.54
9:00	2.55	2.27	0.98
9:20	1.95	2.22	1.29
9:40	1.70	2.00	1.00
April 25,			
8:00 A. M.	1.92	1.57	0.43
8:20	0.75	1.93	0.29
8:40	0.54	1.52	0.27
9:00	0.26	1.43	0.24
9:20	0.11	1.25	0.16
9:40	0.00	1.19	0.10

In connection with the reduction phase we also made two series of examinations to show the progressive changes taking place during this period. For this purpose samples were drawn

from the lower pet-cock at the time of filling and at intervals of 20 minutes from that time to the end of the period. The results of these tests are given in Table 6. Unfortunately on the days selected for these examinations the nitrates in the tank were considerably above the average. The figures are of interest, however, as showing the increase in both the nitrates and nitrites during the first portion of the period and then their falling off. Where these figures have been used in connection with others in the general diagram of the cycle, the average of these two series has been reduced by a constant factor to bring them into agreement with the average of the nine series of tests given in Table 5.

Rate of Discharge of the Filter.—It was of importance in our calculations to know the rate of discharge of the tank throughout the whole period of this discharge. For this purpose we ran the effluent of the tank into our large measuring tank and made readings of the guage in this tank at short intervals. The results are shown in Table 7 and will be given later in graphical form.

TABLE 7.
RATE OF DISCHARGE OF THE FILTER-TANK.

ELAPSED TIME. MINUTES	QUANTITY PASSED IN	
	U. S. Gallons	Per cent of Total
5.....	24	10.9
10.....	48	21.8
15.....	71	32.3
20.....	93	42.3
25.....	114	51.8
30.....	134	60.9
35.....	152	69.1
40.....	168	76.4
45.....	181	82.3
50.....	191	86.8
60.....	208	94.5
75.....	214	97.3
110.....	215	97.7
280.....	220	100.0

SUMMARY OF THE ANALYTICAL DATA. GENERAL CONCLUSIONS CONCERNING THE MODE OF ACTION OF THE CONTACT BED.

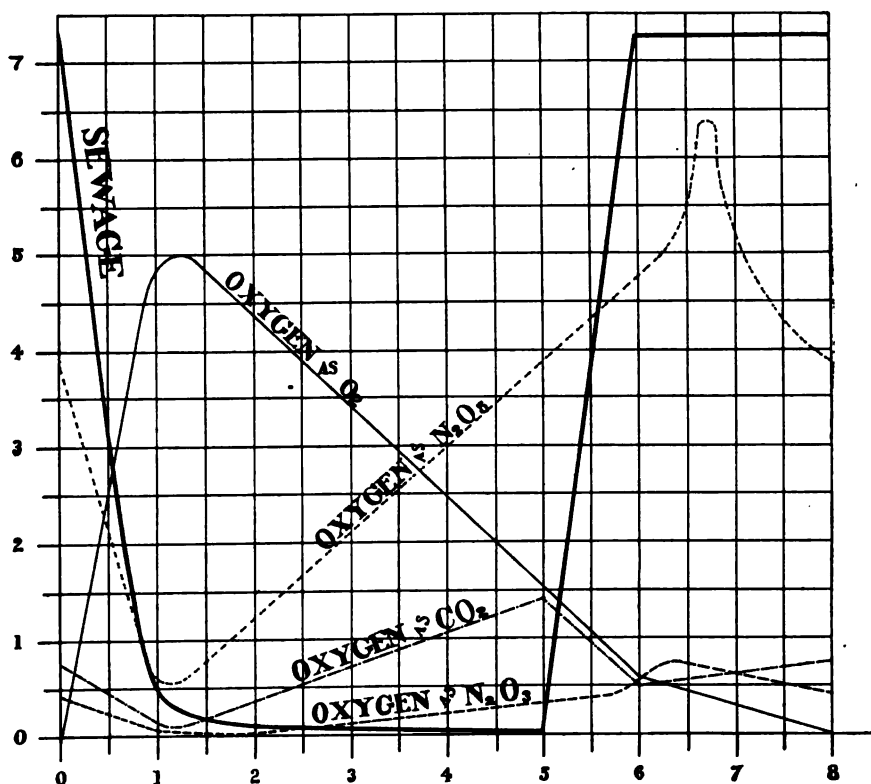
Having now presented in detail the analytical data obtained in our studies of the various phases in the operation of the contact filter, we will attempt to summarize the figures so obtained and to construct from them the complete cycle of this filter. Obviously such a cycle will be ideal and only roughly quantitative. In the actual work of the filter the successive cycles of operation differ from one another to a greater or less degree. Many variables combine to produce this difference, chief of which is the composition of the sewage itself. We are too prone to regard sewage as having a definite and fixed composition, while we know, as a matter of fact, that it varies within fairly wide limits, both as to the amount of the organic matter present and as to the amount and nature of the putrifiable changes which have taken place within it. A second and hardly less important variable to be considered is temperature, which affects directly the biological and chemical activity of the filter-bed, and which also determines to a considerable degree the composition of the sewage. More important than either of these factors, however, seems to be the condition of the filter-bed itself. When such a bed is in good working order it behaves like a delicate organism, is most sensitive to any sudden changes in the environment and exhibits its good days and poor days just as a living organism does.

But in spite of all this variation and instability there are certain reactions occurring in a definite sequence which appear to be characteristic of the process as a whole. These salient features, which it is our purpose to point out and discuss, will become most apparent after we have eliminated as far as possible the variable and less essential features by averaging our entire series of results. We may then construct a complete average cycle of the filter, which, while not representing truly the action of the bed at any one time, will nevertheless serve to show the characteristic reactions of each phase, and the relations of these reactions to one another and of the successive phases to the complete cycle.

In order that the data when thus compiled shall be the more

readily understood, we have constructed from it a diagram representing one complete cycle in the operation. The construction of this diagram we will now describe in detail.

On the axis of abscissas are laid off the times of the various stages starting with the emptying phase, one hour, followed by the



oxidation phase of four hours, the filling phase of one hour, and the reduction phase of two hours. As ordinates are laid off the total weights of the oxygen in its various forms, contained in one liter of the open space of the filter. In order to bring the various curves into the same diagram, the ordinate values refer to centigrams of oxygen in the case of the free oxygen and carbon-dioxide, and to milligrams of oxygen in the case of the nitrates and nitrites. The various lines are obtained as follows: The line

representing the total volume of sewage contained in the filter was constructed from Table 7, showing the rate of discharge, and from the known uniform rate of filling. To avoid confusion the ordinate values are not given for this sewage curve. All the other quantities are expressed in terms of oxygen.

The free oxygen curve starts at 0 with the value of the dissolved oxygen in the effluent. This is the average of 45 results given at the five pet-cocks. At 1, the curve would have the value of one-fifth the total capacity of the tank, this value being based on our analysis of the tank-house air; but for convenience in plotting we have subtracted from this the amount of oxygen which is driven from the tank at the time of filling, also determined by analyses, and which therefore takes no part in the reaction. From 1, the line is arbitrarily drawn straight to the point at 6, at which the value is based upon the measurement of the dissolved oxygen in the sewage as it runs in. The value used is the average of 45 determinations made at the five pet-cocks.

The curves for the oxygen as nitrates and nitrites have values at 0, which show the amounts of oxygen in these forms in the effluent. At 1, the values are calculated from the fact that these substances are passing out with the effluent, and on the assumption that the production of nitrates and the loss of nitrites shown in the next period are taking place in this period at a rate proportionate to the amount of the filtering material exposed to the air. At 5, the values are the amounts in the tank immediately on filling. These values are the averages of the 45 determinations at the five pet-cocks. The curves are then carried on over 6 to the maximum point by means of values given in Table VI, and from this point are drawn as a straight line back to 8, which is the same as 0.

The curve showing the oxygen as carbon-dioxide is of quantitative value only between 1 and 5, where it is based upon the gas analyses made during the empty period. The value at 0 is that of a single analysis of the effluent, and at 6, of one analysis of sewage after flowing in.

A study of this diagram, in connection with certain well-known

physical phenomena leads us to the following conception of the action of the contact filter. As the sewage flows into the bed it passes in thin films over the filtering material which in our case was composed of small crushed stones. The stones are covered on their surface with a thin gelatinous growth which, upon microscopical examination, is seen to be a true zoögleal jelly, containing countless numbers of bacteria. In passing over this gelatinous material the sewage is submitted to adsorption which, we believe, is a very important phenomenon in the action of the bed. Adsorption is that property by which certain substances, notably colloids, will remove dissolved material from its solution. It is well known that colloidal precipitates will adsorb certain bases like ammonia from their solutions and the power of charcoal to remove color and odor from water is another example. By this property a large portion of the dissolved substances is removed from the solution and held on the surface of the filtering material. It is this effect upon which Dunbar and Thumm laid so much stress. We believe that we are the first to definitely explain the manner in which this soluble material is withdrawn from the sewage and retained within the filter until it can be acted upon during the oxidation phase.¹

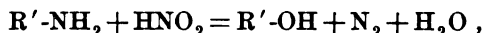
When the tank is full sedimentation takes place as suggested by Dibdin and the suspended solids are largely removed in this way, but it is obvious that adsorption is by far the more important effect.

When the filter is emptied air is drawn in and brought into contact with the gelatinous film with its contained organic matter. The oxygen also is adsorbed, or dissolved, in the film. This fact we showed in our bottle experiments the results of which are given in Table 3. Not only is it thus brought into intimate contact with the organic matter, but by the well known catalytic effects of colloids, the oxidation of the organic matter by the bacteria is thereby very much hastened. In fact it is just conceivable that a part of this oxidation, at least, is due to the bacteria

¹ For a full discussion of adsorption and especially adsorption of substances in solution by colloids, the following papers may be consulted. VAN BEMMELLEN, *Ztschr. f. physik. Chem.* 1895, 18, p. 331; *Ztschr. f. anorg. Chemie*, 1896, 13, p. 233. WALKER AND APPLEBY, *Jour. Chem. Soc.*, 1896, 69, p. 1334.

only in as much as it is the result of a chemical reaction catalyzed by bacterial colloids. The result of this oxidation is the production in some measure of complete oxidation products, nitrates and carbon dioxide, but more important than these are the partially oxidized products. Certain complex molecules like those of proteid substances if exposed to anaërobic fermentations give rise to malodorous products and the process becomes one of putrefaction. But in the presence of a plentiful supply of oxygen it seems to be the case that such substances are decomposed into simpler molecules and that these latter while not completely oxidized are nevertheless stable and inoffensive. About eight-tenths of the total amount of oxygen used up by the filter can only be accounted for by assuming the formation of such partially oxidized bodies.

These partially oxidized substances are carried off by the next dose of sewage. The nitrates and nitrites are dissolved and give rise at this point to what, in our opinion, is one of the most important reactions of the process. The figures given in Table 5 seem to show that the nitrites remain practically constant during the reduction phase and that the nitrates and the free oxygen disappear. What actually happens, we believe, is this. Nitrification, that is the oxidation of ammonia by the nitrifying organism, is going on all the time, using up the oxygen and forming nitrites and nitrates. Another group of bacteria, the denitrifying bacteria, are bringing about the reverse reaction reducing the nitrates to nitrites. The nitrites are being used up as fast as they are formed in a certain characteristic way which we can best explain by the following reaction:



in which R' represents certain monovalent organic radicals. In other words, all primary amines, and many amids and amido compounds react with nitrous acid to form free nitrogen, water and an alcohol. The well known reaction between urea and nitrous acid may serve as an example, and, no doubt, in the case of a very fresh sewage that reaction is one of those which take place.

Since this reaction plays an important part in our theory of the action of the filter we will sum up briefly the evidence for its occurrence.

Amines and amids are well known to result from the decomposition of proteid substances, and the reaction is a necessary one if these bodies are mixed with nitrites.

Careful studies of the applied sewage and the effluents of contact filters generally show a considerable loss of nitrogen during the purification. This loss has in most cases been ascribed to a storage of nitrogen within the filter, and no doubt a portion of it is due to that cause. If, however, as has been pointed out by Clark, we base our nitrogen calculations entirely upon that nitrogen existing as free ammonia, nitrates, and nitrites, then any loss observed must be due to an actual loss of nitrogen during the process, since those three forms of nitrogen cannot, as far as we know, be permanently stored. Moreover, since organic nitrogen is being continually converted into ammonia and often oxidized to higher oxides, the loss of nitrogen calculated by this method will always be a minimum value, or, in other words, the actual loss of nitrogen must be greater than is indicated by such a calculation. On the other hand, if we compare the total nitrogen of the effluent with that of the applied sewage, the difference will be a maximum value for the loss of nitrogen; that is, it will be the actual loss of nitrogen plus the nitrogen stored within the filter. We thus have a means of fixing an upper and a lower limit within which must lie the value denoting the actual loss of nitrogen in any purification process.

In four contact filters studied by him, Clark (1903)¹ found for this minimum value a loss of nitrogen of from 38 to 50 per cent. of the total nitrogen applied as free ammonia. From our analyses already given of the raw sewage and effluent of filter 16 a similar calculation may be made. The filter was a new one, and a certain storage of nitrogen must be looked for. Calculating the results of the first six months, using only the free ammonia and the nitrates and nitrites, we find a loss of nitrogen amounting to not less than 29 per cent of the total applied. A series of deter-

¹ *Loc. cit.*

minations recently made of the total nitrogen in the applied sewage and the effluent of this same filter showed a loss of just 50 per cent of the total nitrogen applied, while calculating the loss from the free ammonia and the nitrates and nitrites only, we get a value of 39 per cent. The one is a minimum and the other a maximum value.

These facts are not new, but we believe that they have never been properly explained or sufficiently emphasized. Many of these substituted ammonias, amines, etc., are volatile substances possessing offensive odors. Their complete removal, therefore, at the very beginning of the process is of great advantage, and here, as we see it, lies the chief value of the nitrates and nitrites. In view of the large amount of nitrogen rendered harmless by this reaction, and of the fact that the nitrogenous substances are responsible for the offensive nature of putrefaction, we cannot avoid the conclusion that the reaction between the nitrites on the one hand and the amines and amido bodies on the other is of prime importance in the contact system of sewage purification. It would seem, moreover, that the reaction in question, accompanied by a large loss of nitrogen, is quite characteristic of this system of purification. Clark¹ finds in the case of those other types of filter in which the aëration is more complete, the sand filter and the trickling filter, that the loss of nitrogen is quite small, about one per cent. Results at this station confirm this fact. Averaging the total nitrogen results from our three sand filters, we obtain as a maximum figure for the loss of nitrogen 5.1 per cent. Similarly for three trickling filters the maximum loss of nitrogen is 6.0 per cent. In the case of our three best contact filters, on the other hand, we calculate from the ammonia, nitrate, and nitrite values a minimum loss of nitrogen of 35.6 per cent, while the maximum value, based on the total nitrogen, is 50.2 per cent.

These differences are too important to be overlooked. They indicate two very distinct types of purification: the slow-sand filter and the trickling filter on the one hand, representing a more or less direct oxidation of the organic matter, comparable with direct

¹ *Loc. cit.*

combustion, and the contact filter on the other, a much more complex process, purifying sewage by means of a reaction peculiar to itself, through which more than one-fourth of the total nitrogen of the sewage is set free in the elementary form.

The slow-sand filter gives rise to completely oxidized products, and, as a measure of its efficiency, it is the practice to compare the amount of nitrates in the effluent with the total nitrogen content of the raw sewage. In the contact filter, on the other hand, complete oxidation is not the result attained. We agree with Dunbar that the appearance of nitrates in the effluent is incidental rather than essential to the process. We believe that they are formed regularly in the operation of the filter and that their formation during the oxidation phase is of much more importance than Dunbar supposes, but we cannot accept the view that their appearance in the effluent is in any way a measure of the efficiency of the purification. In fact, in the light of our experimental results, we think that it may very properly be held, that in any two cases the production of nitrates being equal, the ultimate purification will be inversely as the amount of nitrate in the effluent, or in other words, the stability or non-putrefactive character of the effluent depends in considerable degree upon the amount of nitrate *used up*.

Our conception of the action of the contact filter, therefore, may be summed up as follows:

Nitrification takes place within the bed during the greater part of the cycle, but is of special importance during the oxidation phase. This nitrifying action is essential to the successful operation of the bed.

During the reduction phase nitrification continues at a diminishing rate while denitrification is taking place at an ever increasing rate as the conditions become more and more anaërobic. The interaction between the nitrites and the amines and amido bodies taking place at this time, and the accompanying loss of free nitrogen, is one of the most important reactions in the process and is peculiar to the contact system.

The physical phenomenon of adsorption plays an essential part in the action of the bed, serving to remove soluble matter

from the sewage and to place the atmospheric oxygen in intimate—possibly atomic—contact with the organic matter. The adsorbing material—the bacterial zoöglea—thus acts as a contact catalyzing agent in bringing about an intimate mixture of the reacting substances.

Finally, the quality of the effluent is not dependent upon the amount of nitrates contained within it, although an effluent containing nitrates is certainly a good one. In filters of this type it is the nitrate used up that produces the beneficial result. A perfectly stable effluent may contain no nitrates.

THE DETERMINATION OF THE ORGANIC NITROGEN IN SEWAGE BY THE KJELDAHL PROCESS.

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At the present time there is evident among sewage analysts a tendency to adopt, as a routine procedure in the analysis of sewage and the effluents of sewage filters, the determination of the total organic nitrogen by the Kjeldahl process, either in conjunction with the albuminoid ammonia process of Wanklyn, or as a substitute for that process. The writer in a paper read before the Laboratory Section of the American Public Health Association at the Washington meeting, in 1903, strongly recommended this change and endeavored to show the untrustworthy nature of the so-called albuminoid ammonia figures. The failure of conversion factors to change these figures into even approximate figures for the total organic nitrogen was especially noted.¹

The Kjeldahl process then recommended was one which had been used by Palmer² in his work on the streams of Illinois, and had up to that time given in our work uniformly good results when applied to sewage. When, however, we came to apply the process to our sewage filter effluents we began to get certain suspiciously low results for which we were unable to account. At times the total organic nitrogen was even lower than the albuminoid ammonia figures. These poor results were always on the better grade of effluents. They did not appear regularly, and when they did occur the duplicate analyses never agreed. At about the same time similar difficulties were reported from other laboratories. In one case the raw sewage did not yield reliable results by the simplified Kjeldahl process, and carefully repeated work showed that the poor figures were not due to faulty manipulation but were inherent in the process.

¹ PHELPS, *Jour. Infect. Dis.*, 1903, 1, p. 327.

² *Report on Streams Examinations, Sanitary Dist. of Chicago*, 1902, p. 60.

An experimental study of the Kjeldahl process and its modifications was therefore undertaken in an endeavor to explain the occasional failure of the simplified process which had up to that time been used.

The present paper is a detailed account of these experiments with an analysis of the data obtained. As a result of this work and at the suggestion of the committee of the American Public Health Association on Standard Methods of Water Analysis, a standard method of procedure is outlined which, it is believed, will yield accurate results and which, at the same time, is sufficiently rapid and simple in manipulation to permit its adoption as a routine procedure in sewage analysis.

THE KJELDAHL PROCESS AND ITS MODIFICATIONS.

In general there may be said to be two processes for the determination of the nitrogen in an organic compound. First, the combustion method of Dumas, in which the substance is ignited in the presence of cupric oxide and the nitrogen evolved and measured as such; and, second, the Kjeldahl method or one of its many modifications, in which, by treating with boiling concentrated sulphuric acid, the organic carbon is slowly oxidized and the nitrogen obtained in the form of ammonia. The former process is the absolute one. The latter, owing to its simpler nature, is the one upon which we chiefly rely for the determination of the nitrogen in such mixed materials as fertilizers and food-stuffs, and is the only one which can be used for such a liquid mixture as sewage.

A large number of modifications of the Kjeldahl process have been devised, most of which are intended either to shorten the time necessary for the digestion of the organic matter, or to insure the completion of the reaction. To these ends various reagents are used in addition to the sulphuric acid. Such reagents may be conveniently divided into five groups, according to the work they perform in the determination. First, reagents added to raise the boiling point of the acid; second, reagents which will act as catalytic agents, or carriers of oxygen; third, reducing reagents; fourth, reagents added at the conclusion of the digestion to insure the complete oxidation of certain refractory substances; and fifth,

reagents added in the presence of nitrates either to reduce the nitric nitrogen to ammonia and thus include it in the determination, or else to remove this nitric nitrogen in the form of one of the lower oxides.

As an example of the first group of reagents we have potassium sulphate, as used in the Gunning modification of the Kjeldahl process. This salt raises the boiling point of the mixture and materially lessens the time necessary for complete digestion. Incidentally many reagents added for other purposes are present in sufficient concentration to attain this end.

A number of catalytic agents are employed in the various modifications of the process. Mercury, either as the oxide or in the metallic form, is specified in the official method adopted by the Association of Official Agricultural Chemists (Wiley, 1899).¹ Copper, as the oxide or sulphate, has frequently been used (Jodbauer, 1887);² (Farnsteiner, Buttenberg, and Korn, 1902);³ (Brown, 1903).⁴ Platinum chloride with copper oxide has been recommended by Ulsch, Proskauer and Zülzer (1889).⁵

The addition of reducing reagents has been found necessary in the presence of any considerable quantity of nitrates, and they have also been found to be essential in the analysis of such bodies as amido-azo-benzene, hydroxylamine, and many others (Dyer, 1895).⁶ Zinc dust, salicylic acid, and sugar have been used for this purpose.

Of reagents added at the end of the digestion to complete the process potassium permanganate is most commonly employed. Ward (1894),⁷ showed that there were certain organic compounds, generally those containing a benzene ring, which did not yield to the ordinary treatment, however prolonged, but which were readily

¹ WILEY, U. S. Dept. of Agriculture, Bureau of Chemistry, *Bull. No. 46*, Washington, 1899.

² JODBAUER, *Ztsch. f. anal. Chem.*, 1887, 26, p. 92.

³ FARNSTEINER, BUTTENBERG AND KORN, *Leitfaden für die chemische Untersuchung von Abwässer.* München and Berlin, 1902.

⁴ BROWN, J. W., *Kinetische Studien über katalitsche Beschleunigungen bei der Oxydation von Naphtalin und Analinsulphate durch heisse Schwefelsäure.* Heddleburg, 1903.

⁵ ULSCH, PROSKAUER AND ZÜLZER, *Ztschr. f. Hyg.*, 1889, 7, p. 216.

⁶ DYER, B., *Jour Chem. Soc.*, London, Trans. 1895, 67, p. 811.

⁷ WARD, *The Determination of Nitrogen in the Wet Way.* Unpublished thesis. Massachusetts Institute of Technology, Chemical Department, 1894.

and completely oxidized by the addition of a few crystals of permanganate to the hot acid.

Two kinds of special reagent are used in the presence of nitrates. The first has already been mentioned under the reducing reagents. Such substances as phenol and salicylic acid are nitrated by the nitric acid, and may be subsequently reduced by zinc dust to an amine which will yield ammonia on digestion. Such a treatment will include the nitric nitrogen in the final result. A second method aims to reduce the nitric acid to one of the lower oxides and thus exclude it from the result. For this purpose a mixture of sodium bisulphate and ferric chloride has been used.

EXPERIMENTAL STUDIES.

The failure to obtain as ammonia the total amount of organic nitrogen in the sample of sewage may be due to one or both of two causes: first, the escape of volatile nitrogenous bodies during the evaporation and digestion; and, second, incomplete digestion of the organic matter.

a) *The loss of nitrogen.*—The question of the volatilization of nitrogenous matter during the digestion is a somewhat complicated one. Owing to the basic nature of most of the nitrogenous bodies found in proteid decomposition products and to the non-volatile nature of their salts one would hardly expect any direct loss of the organic matter as such. Some experiments have been carried out, however, to show to what extent we are justified in making such an assumption.

To this end regular Kjeldahl determinations were carried out upon samples of raw and septic sewage, and upon the effluents of various sewage filters. In each case the flask was connected with a Liebig condenser, and in this way all the water of the sample, together with part of the sulphuric acid and any volatile matter which distilled over was condensed. This distillate was subsequently treated with strong alkaline permanganate and redistilled, boiling almost to dryness in order to recover, in large part at least, any organic nitrogen which had escaped during the evaporation and digestion of the sample. The results of these determinations are shown in Table 1.

TABLE 1.
SHOWING THE AMOUNT OF ORGANIC NITROGEN LOST THROUGH VOLATILIZATION DURING THE EVAPORATION AND DIGESTION OF THE SAMPLE.

Sample	PARTS PER MILLION	
	Organic Nitrogen	Nitrogen Recovered in Distillate
Fresh sewage	18.5	0.10
Septic sewage	17.3	0.13
Effluent, contact filter ..	9.0	0.06
Effluent, sand filter	1.7	0.00

These figures seem to indicate that there occur in the sewage and effluents certain organic nitrogenous bodies which are either volatile at the temperature of the boiling liquid or, at least, are capable of being distilled over with the steam. The amount of nitrogen thus escaping may be considered roughly as about one per cent of the total, and this value seems to be fairly constant for the various classes of water studied. It is an entirely negligible quantity, and no method is readily apparent by which the loss can be avoided. In certain abnormal sewages, and especially in waste waters from factories, it is conceivable that a more serious loss may occur in this manner. In any such case it would be advisable carefully to determine this point before proceeding with the nitrogen determination.

There still remains the possibility of the formation of one of the lower oxides of nitrogen, or of free nitrogen itself, during the digestion. In this case there would be a loss of nitrogen which would not be detected in the experiments just quoted, and which, indeed, owing to its very small volume, it would be almost impossible to detect by any direct means. To assume, however, without further evidence that all organic nitrogen, in whatever form it may exist in the molecule, will invariably appear in the form of ammonia after digestion would be quite unwarranted. Fortunately, there is a large amount of experimental evidence bearing out this assumption.

The agricultural chemists throughout the country have made extensive studies of the Kjeldahl process as applied to fertilizers.

In most of this work direct comparisons have been made between this process and the absolute method of Dumas. As a result of these studies, extending over many years, an official Kjeldahl method has been established which is supposed to yield results closely approximating the true nitrogen values. Owing to the somewhat similar natures of the organic matter of fertilizers and that of sewage, this work is of considerable value to the sewage analyst.

In addition to this work upon fertilizers certain exhaustive studies have been made upon the Kjeldahl process as applied to pure substances of known composition. This work all tends to show that under proper treatment the nitrogen of a large number of organic compounds can be recovered quantitatively as ammonia. Dyer (1895),¹ in a study of over 30 compounds, covering a wide range of organic types, obtained satisfactory results in the majority of cases by simply digesting with sulphuric acid in the presence of mercury and potassium sulphate. In certain other cases reducing agents were necessary, and in only one case was he unable to obtain the whole of the nitrogen with a fair degree of accuracy. Sodium nitroprusside gave results about 10 per cent too low. Similar results are reported by Bosshard (1884)² and by Arnold and Wedemeyer (1892).³

It still remains to be shown that in sewage there are no substances which will complicate the reaction and in some way eliminate a portion of the nitrogen. Such a reaction, for instance, as that which occurs between nitrites and primary amines would give rise to a loss of free nitrogen and introduce a serious error in the determination. Three substances may occur in a normal sewage or in the effluents of sewage filters which might possibly lead to such an indirect loss of nitrogen. These are chlorides, nitrites, and nitrates.

There can be hardly any question that these substances might react with the nitrogenous matter of the sewage in such a way as to cause loss of nitrogen. Nitrites may react with amines as indicated above. The possible effect of nitrates is not so readily stated. The present evidence seems to show that the well known

¹ *Loc. cit.*

² *Ztschr. f. anal. Chemie*, 1884, 24, p. 199.

³ *Ibid.*, 1892, 31, p. 525.

rapid destruction of nitrates by sewage is due to bacterial action. (Gayon and Dupetit, 1886;¹ Ampolo and Ulpiani, 1898;² Letts, 1904.³) Chlorides are always present at the same time, and the interaction of the nitric and hydrochloric acids produced will give rise to a certain amount of free chlorine. Experiments made by the writer have shown that, in common with many other oxidizing reagents, free chlorine will cause a loss of nitrogen in the Kjeldahl process. The loss is doubtless due to a reaction similar to that between urea, chlorine and water, viz.:



The question therefore is whether these substances in the quantities in which they are likely to be met with in sewage and sewage effluents are sufficiently concentrated to produce a noticeable loss of nitrogen during the determination. To answer this question the following series of experiments was made.

A series of determinations was first made to study the effect of chlorides. In each case a nitrogen determination was made on the original sample. Enough sodium chlorid was then added to double the original chlorine content and a second determination was made. The procedure followed was the one to be described in another part of this paper as the standard method. The results of the series are shown in Table 2.

TABLE 2.
SHOWING THE EFFECT UPON THE DETERMINATION OF THE TOTAL ORGANIC NITROGEN OF
THE ADDITION OF CHLORIDES TO THE SAMPLE.

SAMPLE	Chlorine	NITROGEN AS			
		Free Ammonia	Albuminoid Ammonia	Total Organic by Kjeldahl, Original Sample	Total Organic by Kjeldahl, After Doubling the Chlorine
Fresh sewage.....	3,600	15.0	3.5	10.0	10.0
Septic sewage.....	1,520	17.5	3.3	8.5	8.5
Effluent, contact filter	4,000	15.0	3.7	7.5	7.5
Effluent, sand filter..	2,700	4.0	0.6	6.0	6.0

¹ *Station Agronomic de Bordeaux*, 1886.

² *Gaz. chim. ital.*, 1898, 28, p. 410.

³ *Sanit. Rec.*, London, 1904, n. s. 33, pp. 426, 536, 552; 34, p. 136.

The excessive chlorine values are caused by the leakage of sea-water into the sewer from which the sewage was pumped. To guard against the possibility that the large amount of chlorid already present in the sample was sufficient to cause the greatest possible loss of nitrogen during the digestion, and that therefore the addition of more chlorid would not increase this loss, the two effluents noted in Table 2 were treated in the following manner. The chlorine of each sample was precipitated by the addition of an excess of solid silver sulphate to the hot sample which had been previously acidified with sulphuric acid. After boiling for a minute, the precipitated silver chlorid was allowed to settle. The nearly clear supernatant liquor was poured off and the silver chlorid was dissolved by prolonged boiling in sulphuric acid. When solution was complete this acid was added to the original solution and the digestion was carried out as usual. The two effluents mentioned gave under this treatment results identical with those given in the table.

It appears therefore that chlorides, *per se*, even when present in such excessive quantities as are found in the sewage used in these experiments, exert no harmful influence upon the determination.

In a similar way the effect of the addition of nitrates and nitrites to the sample was studied. Solutions of these substances were added to the samples in varying amounts and the total organic nitrogen was then determined. These solutions were of such a strength that one cubic centimeter contained one-tenth of a milligram of nitrogen, so that each cubic centimeter added to the sample of 100 c.c. represented one part of nitrogen per million. The composition of these samples and the amount of organic nitrogen found in each case are shown in Table 3.

These results show conclusively that the presence of even considerable amounts of nitrites, nitrates and chlorides does not give rise to any serious loss of nitrogen during the treatment. Taken in conjunction with the results of other workers cited here they seem to indicate that in so far as the nitrogen is released from the organic molecule it can be recovered quantitatively as ammonia. They also show about what accuracy may be looked for

in this process. The process as carried out, does not permit of readings any closer than five-tenths of a part per million, on a sewage or an effluent of poor quality. Such an accuracy is probably all that is warranted, owing to the difficulty of obtaining in

TABLE 3.

SHOWING THE EFFECT UPON THE DETERMINATION OF THE TOTAL ORGANIC NITROGEN OF THE ADDITION OF NITRATES AND NITRITES TO THE SAMPLE.

A.—Sample of Fresh Sewage.

Analysis. Parts per million.

Nitrogen as free ammonia, 18.5; albuminoid ammonia, 3.5; nitrites, 1.5; nitrates, 0.0; chlorine, 8.10.

PARTS PER MILLION		
Nitrogen Added as Nitrate	Nitrogen Added as Nitrite	Organic Nitrogen Found
0	0	5.5
1	0	6.0
5	0	5.0
10	0	5.0
0	1	5.5
0	5	5.5
0	10	5.5
5	10	5.5
10	5	5.5

B.—Sample of Septic Sewage.

Analysis. Parts per million.

Nitrogen as free ammonia, 22.5; albuminoid ammonia, 3.0; nitrites, 0.0; nitrates, 0.0; chlorine, 1.570.

PARTS PER MILLION		
Nitrogen Added as Nitrate	Nitrogen Added as Nitrite	Organic Nitrogen Found
0	0	8.0
1	0	8.0
5	0	8.0
10	0	8.0
0	1	9.0
0	5	8.5
0	10	9.0
5	10	9.0
10	5	8.5

TABLE 3—Continued.

C.—*Sample of a Contact Filter Effluent.*

Analysis. Parts per million.

Nitrogen as free ammonia, 15.0; albuminoid ammonia, 4.4; nitrites, 0.16; nitrates, 5.3; chlorine, 950.

PARTS PER MILLION		
Nitrogen Added as Nitrate	Nitrogen Added as Nitrite	Organic Nitrogen Found
0	0	7.0
3.5	0	7.0
0	0.16	7.0
3.5	0.16	7.1

D.—*Sample of a Sand Filter Effluent.*

Analysis. Parts per million.

Nitrogen as free ammonia, 0.53; albuminoid ammonia, 0.12; nitrites, 0.2; nitrates, 40.0; chlorine, 1,400.

PARTS PER MILLION		
Nitrogen Added as Nitrate	Nitrogen Added as Nitrite	Organic Nitrogen Found
0	0	1.52
40.0	20.0	1.48

a small volume a proper sample of such a heterogeneous mixture. It is undoubtedly this difficulty that gives rise to the irregularity of the results rather than anything inherent in the process. These irregularities are so scattered, however, that it is apparent that the substances added have exerted no influence upon the results.

b) *The digestion.*—We have then to consider the second point mentioned as a possible source of error in the process, namely, incomplete digestion. It is readily apparent that if the digestion is not complete there may be and probably will be some nitrogen left in the organic form at the end of the process which would not be recovered as ammonia. The official method of the agricultural chemists already referred to calls for a digestion until "the contents of the flask have become a clear liquid which is colorless or at at least only a very pale straw color." Other authorities

allow a "pale yellow color." The writer is convinced that in the various interpretations of the proper end-point of the digestion lies much of the difficulty of obtaining correct results. Especially is this the case when potassium permanganate is used at the conclusion of the digestion. This point was shown in a striking way by some experiments made in an attempt to hasten the process of digestion by the addition of oxydizing agents. It was found that the addition of either potassium permanganate, potassium bichromate, or free chlorine previous to the digestion gave rise to a very considerable loss of nitrogen. The following experiments are selected to illustrate this effect in the case of permanganate:

Experiment 1.—Sample, raw sewage.

Parts per million of nitrogen as	{	Free ammonia	-	-	-	-	20.0
		Albuminoid ammonia	-	-	-	-	4.0
		Nitrites	-	-	-	-	0.0
		Nitrates	-	-	-	-	0.0
		Total organic	-	-	-	-	13.5

One hundred c.c. of this sewage was treated with 5 c.c. of potassium permanganate solution (.4 gm. per litre), and a few drops of sulphuric acid and boiled for 10 minutes. It was then treated with sulphuric acid and digested as usual. The organic nitrogen recovered was 8.5 parts per million.

Experiment 2.—Sample, sand filter effluent.

Parts per million of nitrogen as	{	Free ammonia	-	-	-	-	2.4
		Albuminoid ammonia	-	-	-	-	1.4
		Nitrites	-	-	-	-	14.0
		Nitrates	-	-	-	-	20.0
		Total organic	-	-	-	-	7.6

This sample was treated as in Experiment 1. Total organic nitrogen found, 2.6 parts per million. A second portion was warmed for 30 minutes with the permanganate solution. Total organic nitrogen found, 5.6 parts per million.

Similar results were obtained by the addition of potassium bichromate and of aqua regia. The results show clearly that the addition of such oxidizing reagents before the digestion is complete will be accompanied by a decided loss of nitrogen. The

reason for this loss is not clear. In the case of the slow oxidation of the organic molecule by sulphuric acid the carbon is evidently first attacked and the nitrogen is left in the form of ammonia which is incapable of further oxidation either by the acid or by permanganate. In the more violent oxidation of the organic molecule by the permanganate it may be possible that the hydrogen which is attached to the nitrogen is first oxidized, leaving N-O groups which are subsequently liberated and escape as one of the lower oxides of nitrogen.

In order to study the effect of the permanganate upon a sample which is almost completely digested and also to show the comparative results obtained by digesting samples to the different end-points which have been mentioned, the following series of analyses was made. Four samples were used. Upon each sample three determinations of the total organic nitrogen plus the free ammonia were made. In the first case the digestion was carried to a point at which the clear solution had a "pale yellow color," as interpreted by three members of the laboratory staff; in the second case the color obtained was "very pale straw," and in the third case the digestion was carried out until the color was entirely gone. Permanganate was added at the conclusion of each digestion as in the standard procedure. The following results were obtained:

TABLE 4.
SHOWING THE EFFECT OF INCOMPLETE DIGESTION UPON THE AMOUNT
OF NITROGEN RECOVERED.

SAMPLE.	PARTS PER MILLION OF ORGANIC PLUS AMMONIACAL NITROGEN		
	Pale Yellow	Very Pale Straw	Colorless
Fresh Sewage.....	28.5	30.0	30.0
Septic Sewage.....	25.0	26.0	31.0
Contact filter, 16.....	9.5	10.0	11.0
Contact filter, 13.....	10.0	11.0	14.0

It is evident from these figures that a colorless solution must be obtained before the digestion may be said to be complete, and that even the "very pale straw color" is not a satisfactory end-point in the case of most samples.

While it has been shown that the addition of the permanganate at an early stage of the determination will give rise to a loss of nitrogen, yet it is true that the use of this reagent at the conclusion of the digestion, will, in some cases at least, release a certain amount of organic nitrogen which has withstood the action of the acid, giving rise to an increased nitrogen value. Ward (1884),¹ states that compounds containing a benzene ring cannot be completely oxidized by the action of the acid alone, but that the addition of permanganate will always complete the oxidation. In Table 5 is given a list of duplicate analyses by the standard procedure except that on one sample in each case permanganate was not used. It will be seen that in many cases there is no increase in the nitrogen value due to the addition of permanganate, while in others the increase is considerable. The use of permanganate is therefore advisable although it is not essential in all cases.

TABLE 5.

SHOWING THE EFFECT OF THE ADDITION OF POTASSIUM PERMANGANATE AT THE CONCLUSION OF THE DIGESTION UPON THE AMOUNT OF ORGANIC NITROGEN RECOVERED.

Parts per Million

SAMPLE	NITROGEN AS			
	Free Ammonia	Albuminoid Ammonia	Organic Nitrogen	
			With Permanganate	Without Permanganate
Sewage.....	15.0	3.5	10.0	8.0
Sewage.....	20.0	7.1	24.0	19.0
Sewage.....	17.5	6.3	23.0	21.0
Septic sewage.....	22.5	4.2	12.5	12.5
Contact filter Effluent....	12.4	2.1	4.7	4.2
Sand filter Effluent.....	1.6	1.0	2.5	2.5
Septic tank Sludge.....	65.0	22.0	122.0	122.0

The time necessary to accomplish digestion may often be longer than is consistent with the routine analysis of a large number of samples. The question of the use of a catalytic agent has therefore received considerable attention in this study. Of those metallic salts which have been recommended for this

¹ *Loc. cit.*

purpose by various workers copper sulphate was selected as being the one most satisfactory from many points of view. Brown (1903),¹ has shown in a careful quantitative study of the catalytic acceleration of the oxidation of aniline and naphthaline by sulphuric acid that copper and mercury rank first among such catalytic agents and that they are about equal in efficiency. Copper sulphate besides being the cheaper and more convenient substance to use, possesses the additional advantage over mercury that it does not require the addition of sodium sulphid to the solution before distillation. The contrary opinion is often expressed but the writer is satisfied after a careful study of this point that if sodium carbonate is used for neutralization the ammonia can be completely recovered from the solution without the addition of the sulphid.

c) *Minor details.*—As to the amounts of the sample and of the reagents to be employed the attempt has been to employ as large a volume as convenient of the sewage and as small an amount as possible of the other reagents. This practice in the first place allows of a more accurate sampling of the sewage or effluent than if smaller amounts are taken and at the same time reduces the effect of the "blank" due to the reagents to a minimum. I have therefore used, in general, 100 c.c. of the sample and 5 c.c. of sulphuric acid. With these quantities and ordinary pure reagents the blank for the reagents may be entirely neglected.

Having in view the advantages of a process made as simple as possible by the use of few reagents, it has not seemed advisable to attempt to recover the nitric nitrogen by the Kjeldahl process. This may be done if desired by the official agricultural method already cited. In water and sewage work the nitrate determination can be made in a much simpler manner, and indeed would always be so made even if it were also included in the total nitrogen determination. Nitrates will never be found in sewage or sewage effluents in such quantities as to interfere with the process by their own reduction, and this is the chief reason for their determination with the organic nitrogen in fertilizer analysis.

¹ *Loc. cit.*

PROPOSED STANDARD METHOD.

The following is recommended as a standard method of procedure for the determination of the total organic nitrogen in sewage and in the effluents of sewage purification processes.

APPARATUS REQUIRED.

Kjeldahl digestion flasks. These should be of Jena glass and of about 200 c.c. Flasks having an egg-shaped or oval bulb are preferable to those with spherical bulbs, because they will permit of carrying out the digestion in smaller volume without danger of cracking.

Stills. The ordinary indirect stills such as are used in the analysis of sewage, are to be preferred although the direct still may be used when more convenient.

SOLUTIONS REQUIRED.

Sulphuric Acid. Chemically pure sulphuric acid of about 1.83 s.g.

Sodium Carbonate. A saturated solution of sodium carbonate. Live steam is blown through this solution until it gives no test for ammonia with the Nessler solution.

Nessler solution and a *standard solution of ammonium chloride* such as are used in the determination of ammonia.

Copper sulphate and *potassium permanganate.* Chemically pure crystals.

THE DETERMINATION.

One hundred c.c. of the sample are placed in a digestion flask. Five c.c. of sulphuric acid and a small crystal, about 0.1 gm., of copper sulphate are added and the flask is placed over the flame and boiled down briskly until the contents begin to darken. The flame is then turned down until only a gentle simmering occurs. The digestion is allowed to proceed until the yellow color of the contents of the flask has entirely disappeared. With practice this point can be determined in spite of the green color of the copper salt. If any doubt exists the flame may be removed for a minute when the copper salt will at once settle out perfectly colorless. When the digestion is complete the flame is removed and small crystals of permanganate are added one at a time until the green

precipitate is permanent. The flask is then allowed to cool. When cool the contents of the flask are made up to 500 c.c. with water. Ten c.c. (or more, according to the amount of the nitrogen in the sample), are removed, placed in the distilling flask, diluted with an equal volume of ammonia free water, and neutralized by the addition of 10 c.c. (or an amount equal to the amount of the acid mixture taken), of the sodium carbonate solution. This is a large excess and no indicator need be used. Fifty c.c. are then distilled over by blowing steam through the flask, and the

TABLE 6.

ANALYSES OF BOSTON SEWAGE AND OF THE EFFLUENTS OF SEWAGE FILTERS MADE AT THE SANITARY RESEARCH LABORATORY AND SEWAGE EXPERIMENT STATION OF THE MASSACHUSETTS INSTITUTE OF TECHNOLOGY.

SAMPLE	PARTS PER MILLION OF NITROGEN AS					
	Ammonia		Nitrates	Nitrites	Organic Nitrogen	
	Free	Albuminoid			I	II
Fresh sewage...	20.0	6.1	0.5	0.20	13.5	13.0
	20.0	5.3	0.0	0.18	14.0	14.0
	20.0	6.6	0.2	0.0	14.0	14.0
	22.5	7.0	0.0	0.20	13.5	14.0
	23.5	6.5	0.0	0.00	13.0	13.0
	19.5	5.0	0.0	0.20	12.0	11.5
	16.0	5.0	0.0	0.20	11.0	11.0
Septic sewage...				0.16		
	19.0	2.1	0.0	0.0	4.5	4.8
	23.5	2.7	0.0	0.0	5.5	5.5
	25.0	2.7	0.0	0.0	9.0	8.0
Contact filter effluents.....	20.0	3.0	0.0	0.20	3.5	4.0
	23.5	2.1	0.0	0.0	2.3	2.5
	10.0	1.4	0.8	0.10	4.0	4.0
	19.0	4.8	0.0	0.01	11.0	10.0
	25.0	2.0	0.0	0.0	7.5	7.0
	25.0	2.6	0.0	0.20	6.5	7.0
	12.5	3.6	0.0	0.10	15.5	14.5
	15.0	4.2	0.0	0.16	7.0	7.0
Trickling filter effluents.....	22.5	3.7	5.0	0.60	9.0	8.5
	17.5	4.5	0.0	0.04	6.5	5.5
	14.5	3.7	0.2	0.20	8.0	7.5
	17.5	4.2	2.0	0.16	9.5	9.5
	19.0	3.7	6.0	3.0	7.0	7.5
Sand filter effluents.....	0.52	0.12	40.	0.20	1.48	1.55
	0.80	0.24	30.	0.08	1.8	1.6
	1.2	0.30	35.	0.16	2.8	3.0

ammonia is determined in the distillate in the usual manner by nesslerization.

CALCULATION.

With samples containing from 10 to 40 parts per million of ammonial and organic nitrogen 10 c.c. of the diluted acid mixture is the proper amount for distillation. The distillate may be directly nesslerized. If the ammonia standard used has the usual strength (1 c.c. = 0.00001 gm. nitrogen), then each cubic centimeter of the standard used represents five parts per million of nitrogen in the sample. Multiplying the reading by five and subtracting from the product the free ammonia value, the result is the total organic nitrogen, expressed in parts per million.

The analyses of sewage and of the effluents of sewage filters of various types which have been made at the Sanitary Research Laboratory and Sewage Experiment Station of the Massachusetts Institute of Technology are given in Table 6. The sewage used is drawn from the trunk sewer of the Boston Main Drainage system.

TABLE 7.

SAMPLE	PARTS PER MILLION OF NITROGEN AS			
	Free Ammonia	Albuminoid Ammonia	Total Organic	
			I	II
Boston—				
Albany St. sewer*.....	3.0	5.3	18.5	18.0
Dartmouth St. sewer*.....	4.0	7.3	22.0	22.0
Brockton, Mass.—				
Stale sewage.....	45.0	8.0	17.5	17.5
Sewage sludge.....	75.	225.	415.	420.
Effluent, sand filter.....	0.24	0.12	0.62	0.60
Concord, Mass.*.....	9.0	5.0	16.0	15.5
Lawrence, Mass.—				
Sand filter No. 1.....	0.068	0.064	0.40	0.43
“ “ “ 5.....	0.73	0.69
Pride's, Mass.—				
Private septic tank. Effluent	21.0	6.6	11.5
Worcester, Mass.....	37.5	7.7	22.5	22.5

* Small sewers. Very fresh sewage.

The analyses are given to indicate the probable accuracy of the method proposed and to show the rough relation existing between the total organic nitrogen and the albuminoid ammonia.

To test more fully the proposed method certain samples were collected from outside sources and submitted to analysis. The writer is indebted to Professor L. P. Kinnicutt, Mr. H. W. Clark, and Mr. G. E. Bolling for assistance in the collection of samples from Worcester, Lawrence, and Brockton respectively. A description of these samples and the analytical results obtained are given in Table 7.

TESTS OF A METHOD FOR THE DIRECT MICROSCOPIC ENUMERATION OF BACTERIA.

C.-E. A. WINSLOW AND G. E. WILLCOMB.

(From the Sanitary Research Laboratory and Sewage Experiment Station of the Massachusetts Institute of Technology.)

A METHOD for the direct microscopic enumeration of bacteria in sewage was devised by one of us (C.-E. A. W.), during the summer of 1903. The development of the method and the results of its application to sewage effluents are fully described elsewhere (see p. 41). In general, however, it may be said that the microscopic count indicated the presence of from 10 to 100 times as many bacteria as develop upon the gelatin plate, or even upon the Nährstoff agar medium. This result was so striking that it seemed to us worth while to study, in a little more detail, the relation between the counts obtained by the visual and the cultural methods, with a view to determining the real significance of the higher numbers apparent under the microscope and the practical value of the new process of enumeration.

The microscopic method as finally developed is as follows: After the usual shaking, a sample is withdrawn from the bottle in a sterile graduated one c.c. pipette and one-twentieth of a c.c. is allowed to flow upon a clean square cover-slip. Cover-slips should be boiled in a 10 per cent solution of potassium bichromate in 50 per cent sulphuric acid, and allowed to lie in this cleaning mixture. Just before using they may be rinsed in 50 per cent alcohol and dried on a silk cloth, not in the flame. One-twentieth of a c.c. of water placed on such a cover-slip spreads evenly and should be allowed to dry in the air without too sudden heating. After drying, it is fixed by passing through the flame, covered with Ziehl-Nielsen's carbolfuchsin, warmed till steam just rises, washed, dried, and mounted.

For counting the bacteria we used a Sedgwick-Rafter eyepiece micrometer, made for the study of the larger micro-organisms in drinking water. It bears a large square divided

into quarters, one of which is further subdivided into twenty-fifths. With the $\frac{1}{8}$ -inch oil immersion objective and a one-inch eyepiece we were able so to adjust the draw-tube that one side of the large square should equal .1 mm. on the stage, its area thus being .01 sq. mm. With a cover-slip, 22 mm. on a side, and an area of 484 sq. mm. one square covered $\frac{1}{484}$ of the total area, or $\frac{1}{50000}$ approximately. In practice we have generally counted 10 fields in different parts of the cover-slip, selecting them in a line passing from one corner diagonally to the middle of the opposite side and then back to the other corner of the side first taken. The total of 10 fields must be multiplied by $\frac{50000}{10} \times 20 = 100,000$ to give the number of bacteria per c.c. of the original sample.

The difficulties of manipulation in this process we have not found serious after a little practice. With properly cleaned cover-slips fairly even distribution may be secured. For example, the results of the enumeration of 10 squares in the analysis of three samples of sewage effluent containing widely varying numbers of bacteria are shown in Table 1.

TABLE 1.
NUMBER OF BACTERIA COUNTED IN 10 AREAS OF .01 MM. ON A 22 MM.
COVER-SLIP.

Sample.			COVER-SLIP.									
Septic Tank	- -	52	68	56	48	52	64	68	60	64	72	
Contact Filter	- -	12	8	20	20	12	16	12	12	28	4	
Sand Filter	- - -	4	0	10	11	9	7	9	5	10	6	

The sampling error with so small a portion as .05 c.c. is, of course, considerable; and in order to measure it we made analyses of several samples in duplicate, flooding each cover-slip with a separate portion taken from the bottle of sewage with a distinct pipette. The results, as shown in Table 2 indicate that the sampling error may be over 15 per cent; but this is not a bad showing by comparison with the results obtained by the plate method, when fluids containing large numbers of bacteria and much suspended matter are diluted several times.

With practice the microscopic count may be made with considerable rapidity. The sampling of 10 sewages and effluents and the spreading of the samples on the cover-slips take about

20 minutes; the staining and mounting after two hours' drying in the air take 15 minutes, and the counting 30 minutes. The actual time devoted to the analysis of each of the 10 samples is thus less than seven minutes. The fact that results can be reached in one or two hours, instead of 24, might also prove of importance under certain conditions.

TABLE 2.
VARIATIONS IN NUMBER OF BACTERIA IN SAMPLES OF SEWAGE AND EFFLUENT
ANALYZED IN DUPLICATE.

Sample	Number of Bacteria Counted in 10 Squares	Average	Average Deviation	Average Percentage of Error
Trickling Filter	180 148 198 168 148 168	168	12	7.
Septic Tank...	280 292 240 232 262 152 244 172 280	236	36	15.
Sewage	544 876 528 652 628 716 624 680 468	636	84	13.

Obviously the microscopic method of counting is applicable only to substances containing considerable numbers of bacteria. When the number of fields to be examined is very large the process becomes both tedious and inaccurate, and it will probably not be useful for the counting of bacteria in numbers less than 25,000 per c.c.

The high numbers of bacteria found by this method as compared with the ordinary plate count might be due to any or all of three causes. First, even the most careful shaking fails wholly to separate chains and groups of organisms into their constituent cells; hence colonies on a plate sometimes represent not individuals but pairs or larger aggregations of bacteria. Second, the presence of the dead bodies of bacteria in a stainable condition would make the microscopic count larger than the plate count. Third, there may be present organisms which do not grow upon our ordinary nutrient media, or representatives of groups ordinarily appearing which, though still living, are too weak to thrive under the peculiar conditions of a solid culture. With respect to the first and third factors the microscopic count is superior to the plate count, while with regard to the second it fails to give a correct idea of the total number of living bacteria

present. Upon the relative importance of these three factors then the value of the microscopic method must depend.

The extent to which unseparated groups of bacteria introduce an error in the ordinary plate count depends, of course, largely upon the thoroughness with which shaking is carried out. In our first experiments with pure cultures we found very marked differences between the microscopic count and the plate count, due mainly to this cause. This was particularly the case when a suspension was made from the surface growth upon some solid medium, since in this case the bacteria occur in close aggregates very hard to break up. Table 3 illustrates this point.

TABLE 3.
COMPARISON OF MICROSCOPIC COUNT AND PLATE COUNT. AQUEOUS SUSPENSIONS OF *B. SUBTILIS* FROM AGAR STREAK KEPT AT 20°.

Number of hours from beginning of experiment...	0	3	7	24	32
Bacteria per c.c. microscopic count	2,300,000	1,000,000	1,500,000	9,000,000	5,600,000
Bacteria per c.c. gelatin plate	450,000	260,000	540,000	3,000,000	1,000,000

TABLE 4.
COMPARISON OF MICROSCOPIC COUNT AND PLATE COUNT. *B. SUBTILIS* IN DILUTED BROTH.

Number of hours from beginning of experiment		0	6	24	48	72
A. at 20°	Bacteria per c.c. microscopic count...	4,500,000	9,300,000	21,000,000	14,500,000	11,900,000
	Groups of bacteria, per c.c. microscopic count	3,000,000	8,000,200	15,000,000	6,800,000	9,000,000
	Bacteria per c.c. gelatin plate.....	3,000,000	7,000,000	500,000	1,000,000	6,500,000
B. at 15°	Bacteria per c.c. microscopic count....	5,000,000	10,200,000	6,300,000		
	Groups of bacteria per c.c. microscopic count.....	3,600,000	6,200,000	4,500,000		
	Bacteria per c.c. gelatin plate.....	7,000,000	770,000	2,500,000		

Table 4 shows the comparative counts obtained by diluting a broth culture with water. Here the results obtained are much better, with the exception of three instances in which the gelatin

count was abnormally low. Separate counts are recorded for the individual cells and for the groups of bacilli under the microscope, and they show that the number of cells was from 10 to 100 per cent higher than the number of groups. In Table 10 the same thing is shown in more detail. In this experiment suspensions were made in diluted broth of streak cultures of *Micrococcus roseus* and inoculated with a small portion of surface growth from a streak of *B. subtilis*. From the table it appears that in spite of very thorough shaking both cocci and bacilli adhered so closely together that the number of cells was often double the number of groups. In the examination of sewage and sewage effluents by the microscopic method it is common to find pairs and even long chains of cocci and bacilli, and irregular groups of organisms held together by a sort of zoöglöea. In this respect the microscopic count gives a truer idea of the number of bacteria present than does the plate method.

The presence in the fluid to be analyzed of large numbers of dead bacterial cells in such a condition as to be fixed and stained by carbolfuchsin would, on the other hand, introduce a serious error in the microscopic count; and this possibility appears to us the most serious theoretical objection to the method. We know, indeed, from the study of higher organisms that protoplasts after death degenerate and break up so that definite cell structures can no longer be made out by treatment with anilin dyes. Whether under the conditions which obtain in organic fluids this process is sufficiently rapid to prevent the accumulation of a large number of stainable dead cells is the question upon which the value of the microscopic count depends.

The fact that dead bacteria do lose their staining property is made clear by the examination of any old solid culture. We have, for example, made a suspension from an old culture of *B. typhosus*, which, when stained and mounted, showed the field crowded with rods so faintly tinged as to be barely visible. On the other hand, under certain conditions, dead cells may retain their normal condition for a considerable period. In a sterilized pure culture, for example, the bacteria are still stainable after many hours, as shown in Table 5.

TABLE 5.

MICROSCOPIC COUNTS OF AQUEOUS SUSPENSION OF *B. SUBTILIS* BEFORE AND AFTER HEATING TO 70°-80° FOR 30 MINUTES.

Time	Before Heating	After Heating	2 Hours After Heating	4 Hours After Heating	6 Hours After Heating	24 Hours After Heating
Bacteria per c.c. (microscopic count).....	23,900,000	19,200,000	22,500,000	28,900,000	20,100,000	20,900,000
Bacteria per c.c. agar, 37°	3,380,000	19	0	0	0	0

Sterilization, by heat and by most chemical agents, however, probably involves a coagulation of the protoplasm, thus in a way fixing and protecting it from the normal changes of decomposition. In this respect the test is not a fair one; and we next used a suspension in which the bacteria were killed by exposure to direct sunlight. Again the dead cells persisted for many hours as shown in Table 6.

TABLE 6.

MICROSCOPIC COUNT OF AQUEOUS SUSPENSION OF *B. SUBTILIS* BEFORE AND AFTER EXPOSURE TO DIRECT SUNLIGHT.

Time	Before Exposure	After 2 Hours' Exposure	After 5 Hours' Exposure	20 Hours After Removal from Sunlight
Bacteria per c.c. (microscopic count)	22,000,000	27,200,000	29,600,000	15,400,000
Bacteria per c.c. (agar, 37°) ..	950,000	147	60	770,000

Still our conditions did not duplicate those which actually obtain, since in sewage and other decomposing fluids dead bacteria occur in the presence of other living and actively multiplying cells; and the products of growth of the latter might be expected to exercise an important effect upon the dissolution of the former. We first tested this point by sterilizing septic sewage in the autoclave and mixing with it one-tenth its volume of untreated septic sewage. Thus a large number of dead cells were brought into contact with a smaller number of living bacteria under such conditions that the latter could multiply freely. The results shown in Table 7 indicate a destruction of three-fifths of the total

of living and dead bacteria present during the first three hours after the samples were mingled signifying the dissolution of 30,000,000 cells per c.c.

TABLE 7.

MICROSCOPIC COUNT OF BACTERIA IN MIXTURE OF SEPTIC SEWAGE WITH TEN VOLUMES OF STERILIZED SEPTIC SEWAGE.

Number of hours from beginning of experiment..	0	3	6	24
Bacteria per c.c.....	45,000,000	21,000,000	19,000,000	61,000,000
'Duplicate count).....	54,000,000	17,000,000	15,000,000	39,000,000

The crucial test of the microscopic count could not be made with sewage since with a mixture of various germs the presence of forms incapable of development in our nutrient media excludes the possibility of a control by the plate method. We therefore next examined pure cultures of certain bacteria which grow readily on the gelatin plate. Table 8 shows the result of parallel cultural and microscopic counts of *B. megatherium*; and the agreement is in most cases quite as good as could be obtained between two plate counts of fluids so rich in bacterial life.

TABLE 8.

COMPARISON OF MICROSCOPIC COUNT AND PLATE COUNT.
B. MEGATHERIUM.

NUMBER OF HOURS AFTER BEGINNING OF EXPERIMENT		BACTERIA PER C.C.	
		Microscopic Count	Gelatin Plate
Peptone at 37°	0.....	104,000,000	123,000,000
	17.....	16,400,000	17,200,000
	41.....	144,000,000	134,000,000
	65.....	48,000,000	17,600,000
Broth at 10°	0.....	8,000,000	1,060,000
	53.....	20,000,000	25,800,000
	72.....	26,700,000	34,800,000

In the first experiment of Table 8, there twice occurred a marked falling off in the number of bacteria, 100,000,000 per c.c. disappearing in each case in about 24 hours. With a view to comparing more fully the value of the microscopic count with

increasing and with decreasing numbers of bacteria we examined in parallel, cultures of *B. megatherium* in water at 20° and in Dunham's peptone solution at 27°. In water the bacilli rapidly decreased and in peptone solution they multiplied; in general the agreement of the two methods of counting is very close, only four wide deviations occurring in the whole table, in one of these the gelatin count being in excess. Losses of 200,000 bacteria out of 250,000 and of 112,000,000 out of 116,000,000 in four hours are correctly registered by the microscopic method as well as numerous less marked diminutions.

TABLE 2.

COMPARISON OF MICROSCOPIC COUNT AND PLATE COUNT. *B. MEGATHERIUM* IN WATER AT 20° AND IN PEPTONE SOLUTION AT 27°.

NUMBER OF HOURS AFTER BEGINNING OF EXPERIMENT	BACTERIA PER C.C.			
	IN WATER		IN PEPTONE SOLUTION	
	Microscopic Count	Gelatin Count	Microscopic Count	Gelatin Count
0.....	254,000	157,950	250,000	187,200
48.....	245,000	200,000	283,000
52.....	50,000	950,000
96.....	0	1,000	1,550,000	500,000
119.....	0	0	2,335,000
0.....	1,200,000	1,220,000	1,150,000
1.....	950,000	13,400	1,350,000	1,345,000
22.....	550,000	530,000	1,600,000	1,550,000
43.....	350,000	540,000	4,000,000	4,100,000
0.....	435,000	380,000	435,000	520,000
20.....	8,000	2,195,000	13,764,000
25.....	15,000	2,000	4,030,000	4,590,000
44.....	0	1,840	580,000,000	255,200,000
47.....	6,500	3,060	340,000,000	298,840,000
51.....	600,000,000	126,380,000
0.....	116,000,000	100,000,000	116,000,000	110,000,000
4.....	4,600,000	3,400,000	2,000,000	2,500,000
28.....	34,000,000	29,000,000

Finally we carried out a series of experiments with mixed cultures of two bacteria, *B. subtilis* and *M. roseus*, which could be easily separated both on the plate and under the microscope, in order to study the interaction of two distinct species. Both individual cells and groups of each form are tabulated separately

in Table 10 with gelatin plate counts of both in the last two series. In the first three experiments the colonies of *M. roseus* could not be counted because with inadequate dilutions the bacilli liquified our plates before the cocci developed.

Again the plate counts and the groups noted under the microscope correspond very closely except with regard to the bacilli in the last two experiments which showed low numbers on gelatin. In this particular case it is apparently the plate method which was in error since the steady increase noted under the microscope could only have been conditioned by a corresponding multiplication of living germs. While the bacteria increased, the cocci for which 37° is too high a temperature, showed a general decrease. In the first experiment nearly 2,000,000 disappeared in six hours, in the second, 22 million in two hours; in the fourth 225 million out of 675 million in two hours; in the fifth, 100 million out of 250 million in two hours.

TABLE 10.

COMPARISON OF MICROSCOPIC COUNT AND PLATE COUNT. MIXED CULTURE OF *B. SUBTILIS* AND *M. ROSEUS* IN DILUTED BROTH AT 37°

Number of Hours from Beginning of Experiment	Bacilli per c.c. Microscopic Count	Groups of Bacilli per c.c. Microscopic Count	Bacilli per c.c. Gelatin Plate	Cocci per c.c. Microscopic Count	Groups of Cocci per c.c. Microscopic Count	Cocci per c.c. Gelatin Plate
0.....	2,700,000	4,000,000	1,800,000
6.....	70,800,000	22,000,000	0
0.....	1,200,000	1,200,000	1,000,000	18,900,000	7,800,000
2.....	7,200,000	5,700,000	2,500,000	22,800,000	7,700,000
4.....	50,700,000	26,700,000	30,000,000	0	0
0.....	3,900,000	1,800,000	3,000,000	322,500,000	148,800,000
2.....	3,900,000	2,700,000	5,000,000	344,000,000	137,000,000
6.....	30,400,000	12,000,000	6,000,000	396,800,000	184,400,000
8.....	31,500,000	18,000,000	12,000,000	381,000,000	223,000,000	231,000,000
0.....	1,800,000	1,200,000	660,000	697,000,000	277,000,000	231,000,000
2.....	6,300,000	3,900,000	2,000,000	673,000,000	226,000,000	238,000,000
6.....	45,000,000	13,800,000	670,000	454,000,000	200,000,000	217,000,000
24.....	153,000,000	88,500,000	396,000,000	144,000,000
0.....	5,400,000	3,000,000	80,000	178,200,000	76,000,000	70,000,000
2.....	9,000,000	5,700,000	2,000,000	244,000,000	128,000,000	77,000,000
4.....	16,800,000	9,000,000	2,000,000	154,000,000	91,000,000	77,000,000
6.....	37,000,000	23,000,000	4,000,000	148,000,000	94,000,000	84,000,000
24.....	63,000,000	27,000,000	1,000,000	186,000,000	88,000,000	106,000,000
30.....	151,000,000	72,000,000	126,000,000	73,000,000	133,000,000

Altogether the results of our experiments show that with cultures of germs which flourish on the ordinary nutrient media the

microscopic count and the plate count closely correspond, whether the numbers present be increasing or decreasing. Under the latter condition very large numbers of bacteria, 100,000,000 or more to the c.c. may perish from various natural causes and leave no trace discernible under the microscope. The conclusions drawn from the study of cultures sterilized by artificial procedures which may alter the character of the bacterial cell are therefore not applicable to ordinary conditions. There appears to be no appreciable error due to the presence of dead cells when the microscopic count is applied under varied conditions to organisms which grow on nutrient media; and there is therefore no reason to suppose that such an error will affect its results in the examination of fluids containing organisms of other sorts.

If we are justified in this view the difference between the number of bacteria in sewage, as determined by the plate method and the microscopic count, must depend mainly upon the third of the three factors suggested at the beginning of this communication, the presence of organisms which do not thrive on our ordinary nutrient media. This factor cannot well be measured; but its importance must be great when we remember that some spirilla, the nitrifying organisms and many parasitic bacteria, never appear upon our plates. Heyden's Nährstoff medium, with many substances, shows ten times as many bacteria as the gelatin plate; yet, at least two of the classes above mentioned find this substratum totally unfitted for development. In the microscopic counting of sewage samples many of these forms are at once apparent, spirilla, for example, and very large swollen bacilli, and the groups of variable short rods lying in irregular masses of zoöglæa so characteristic of the nitrifiers. Again, of the bacteria which do grow in gelatin, many, like some of the cocci, develop colonies so slowly that they do not appear for four days or more and are practically never counted in our routine analysis. Even among the ordinary metatrophic species individuals are often present which are too feeble to form colonies on solid media; for example, it is a common phenomenon in water analysis for dextrose broth tubes to yield a positive test for *Bacillus coli*, when agar plates inoculated from the same sample give no colon-like colonies.

Our ordinary quantitative bacteriological methods yield satisfactory comparative results whose value has been proved by long and varied application. The number of colonies appearing on the gelatin plate corresponds pretty closely to the amount of decomposing organic matter in a water, and for ordinary sanitary purposes nothing is likely to supplant this method for quantitative analyses. In many instances, however, a knowledge of the number of bacteria of all types might prove of advantage. How wide the application of such a new method may be can only be determined by the detailed study of various substances in connection with new sanitary problems as they arise. In work on bacteriolysis it offers unique advantages for observing the dissolution of the bacterial cell. In milk analysis it has been suggested that it might find application. Wherever it is desirable to know the grand total of bacteria present we believe that this process deserves recognition. In fluids containing 25,000 or more bacteria to the c.c. enumeration under the microscope is rapid, easy, and accurate; and it measures the absolute number of microorganisms present with far greater precision than any other process.

REPORT OF THE COMMITTEE ON ANTITOXIC AND IMMUNIZING SERA.

AS THE Laboratory Section of the American Public Health Association stands for uniformity of method in all routine laboratory tests, it seemed desirable to your committee to take up and consider for its present field of work the application of this principle to the routine testing of antitoxins.

It was deemed wisest first to undertake work on the standardization of the method of testing diphtheria antitoxin, leaving those for tetanus antitoxin and other immune sera for such action as their future therapeutic standing would seem to warrant.

As the subject deals with reactions governed by laws which are at present imperfectly understood, and as the results of these reactions are made manifest only through the use of animals, thereby introducing many unknown factors, it is clearly evident to all that any standard test will be a purely arbitrary one, and that the results obtained by its use can be considered only to approximate accuracy and uniformity.

It is not our intention to reconsider or discuss in detail the investigations of Behring, continued by Ehrlich and his followers in their extensive work, which resulted in the formulation of the so-called Ehrlich Standard Unit for diphtheria antitoxin. A brief review of the events leading up to it will suffice.

Prior to 1897 the test generally applied for the determination of the antitoxic strength of sera was based on the formula arbitrarily chosen by Behring to represent the unit for diphtheria antitoxin. This may be defined as follows: An antitoxic unit is 10 times the amount of antitoxin required to leave intact a 250 gram guinea pig after the injection of 10 times the certainly fatal dose of the toxin,* for pigs of this weight. The method employed was to mix 10 times the minimum dose of a toxin fatal to a 250 gram guinea pig with different quantities of antitoxic serum, and to inject the same subcutaneously into guinea

*This was the ordinary fresh toxin produced in the Behring Laboratory.

pigs of the required weight. That amount of serum which apparently neutralized the pathogenic action of this amount of toxin contained one-tenth of an antitoxic unit. In other words, 90* to 100 minimum fatal doses of the usual diphtheria toxin were to be neutralized by one unit of antitoxin.

Ehrlich in 1896 found that this was not always the case. By using various toxins, and the same toxin at different periods, he observed that a unit of a given serum did not neutralize the same number of fatal doses of such toxins but that the latter varied within such wide limits as from 30 to 130 fatal doses. On the other hand he found that the power of a given toxin to combine with an antitoxin remained constant within narrow limits, and he was led to establish a standard antitoxin in place of a standard multiple of the minimum fatal dose of toxin. Such a standard antitoxin is prepared by him under such elaborate precautions as he believes will insure its permanency, and from time to time, at present every two months, portions of the same are sent out dissolved in a mixture of glycerin and salt solution, for the purpose of enabling producers of antitoxin to standardize such toxins as they desire to use in testing the strength of antitoxic sera. One c.c. of a given dilution of this standard serum represents, according to Ehrlich, one unit of diphtheria antitoxin.

This standard unit of antitoxin has been adopted officially by the German government, and has been used as the standard by the official or prominent laboratories in France, Austria, Denmark and England, in Europe, and by the state laboratories in Massachusetts and New York, and the majority, at least, of the private producers of antitoxin in America. Your committee, therefore, will not enter into any discussion or consideration of either the theoretical or practical problems connected with the establishment of this unit, but will pass on to the discussion of the methods for testing the specific strength of diphtheria antitoxins by the use of this standard antitoxic unit or of the Standard Serum issued by the United States Public Health and Marine Hospital Service.

In a general way the procedures are as follows:

*Some fraction of the 10 fatal doses was neutralized by the guinea pig.

If we have at hand either of these standard sera, it becomes necessary for the standardization of a toxin to add to that amount of a dilution of a standard serum which contains exactly one unit, such an excess of toxin that the resulting mixture will just prove fatal to a standard weight guinea pig on the third or fourth day after its subcutaneous injection. This point Ehrlich denominates L+. The amount of toxin which would be needed to neutralize completely the antitoxin he designates L0. Both these terms are now commonly employed and are recommended to be used in designating these points. Having determined the L+ dose of a given toxin, that amount of any antitoxic serum which when mixed with this dose will prevent the death of a standard weight guinea pig before the fourth day after injection contains one unit of antitoxin.

From time to time members of your committee have conferred with one another concerning the methods used by each one in the application of both the tests for the determination of the L+ dose of toxins by the use of the Standard Serum and the estimation of the antitoxic strengths of sera therefrom. These consultations led to a certain slight degree of uniformity in the methods used, but it has seemed desirable to several members to have the various methods discussed, and one, uniform at least along general lines, adopted as a standard.

One of the members of the committee, having observed that the results of his test on a given toxin did not agree with those of a co-worker on the same toxin, decided to ascertain how far uniform the results obtained by different investigators in different laboratories, and under their varying environment, might be. To this end he requested samples of toxins from seven laboratories, which he tested with the following results:

TOXIN A.—L+ dose said by sender to be 0.32 c.c.

Tested for 0.33 guinea pig died in 27 days.

"	"	.34	"	"	"	"	"
"	"	.35	"	"	"	"	"
"	"	.42	"	"	"	6	"
"	"	.43	"	"	"	4	"

TOXIN B.—L+ dose said by sender to be 0.76 c.c.

Tested for 0.76 guinea pigs all died in 4 days.

TOXIN D.*—L+ dose said by sender to be 0.33 c.c.

Tested for 0.33 on a large number of guinea pigs at different periods and all died within four days.

*Toxin C had not been tested by Ehrlich's method.

TOXIN E.—Same as D, with same results.
 TOXIN F.—L+ dose said by sender to be 0.19 c.c.
 Tested for 0.19 guinea pigs died in 48 hours.
 " " 0.185 " " " " 4 days.
 " " 0.18 " " " " 4 "

Subsequent to these tests it was deemed advisable for some one member to submit samples of an antitoxic serum to the other members for the purpose of obtaining the results of tests made by each member using the methods regularly employed for this work in his laboratory. These results are presented in the following tables. Each table represents some of the tests applied by one member. With but one exception the method of ascertaining the L+ dose of toxin by the use of the Ehrlich Standard Serum, and of testing the serum against that dose, was employed. In this case the former method of Behring, modified so that 90 minimum lethal doses of a fresh toxin are neutralized by one unit of antitoxin, was employed. The results in those tests were as follows:

TABLE 1.
 BEHRING'S TEST MODIFIED.

Toxin 90 M.L.D.+1/300 c.c. Serum (300 units per c.c.) Animal lived.
 " " " " 1/325 c.c. " (325 " " ") " died 4½ days.
 " " " " 1/350 c.c. " (350 " " ") Animal died 4 days.
 " " " " 1/350 c.c. " (350 " " ") Animal died 3½ days.

TABLE 2.
 EHRLICH'S METHOD.

DATES	L+ TESTS WITH EHRLICH'S SERUM		L+ DOSE USED AGAINST SERUM TESTED	NUMBER OF UNITS PER C.C. TESTED FOR					
	For	Result		300	310	320	325	330	350
May 7.....	.23 c.c.	-8*							
	.24 "	-7							
	.24 "	-6							
	.25 "	-5							
	.25 "	-3							
	.26 "	-3							
July 9.25 "	+							
	.275 "	+							
July 16.28 "	-4							
	.3 "	-3							
	.32 "	-2							
Sept. 7.27 "	-3							
	.28 "	-3							
	.29 "	-3							
Sept. 13.26 "	+	.26	+	+	-3	-3	-3	-2

* The interpretation of the signs in all the tables should be as follows:
 + = Animal lived.
 - = " died.
 Numerals = Days after injection on which animal died

REPORT OF COMMITTEE ON

TABLE 3.
EHRlich's METHOD.

DATES	TECH- NIQUE	L + TESTED FOR OR USED	NUMBER OF UNITS PER C.C. TESTED FOR				
			232.5	275	287.5	300	350
August 31.....		.53 + .53 + .54 -5					
September 3.....		.54 + .55 -2 .55 -2 .56 -3 .56 -2					
September 6.....		.545 -3 .545 -4					
September 13.....	1 2 3 3	.545 .545 .545 .545				++ -4+ -3 + -4+ ++ -4 +	-3+ -4+ -3 -3 -2+ -3+ -2 -2
September 19.....	3 2* 2* 3	.545 .545 .545 .545		++ ++ -4+ -4+ -4+ ++	-4 + -4 -3 -3 -4		
September 27.....	1 2 3	.545 .545 .545		++ ++ ++ ++ ++ ++	-4+ -4+ ++ ++ ++ ++		
October 10.....		.545 -3 .545 -4 .55 -3 .55 -3	++ ++ ++ ++	+ + + +	++ ++ ++ ++		
October 18.....	3				+ +		

Technique No. 1. Ordinary 1 c.c. pipettes and ordinary syringes.
 2. Dilutions made in titrated flasks. Ordinary syringes and Ehrlich pipettes used.
 3. Ehrlich pipettes and Rosenau injecting syringes used.

*Slight alterations in technique were made in these tests.

†Dilutions in these tests made at 15° C. All others at 23°-25° C.

TABLE 4.
EHRlich's METHOD.

DATE	L + TESTS WITH EHRlich's SERUM		L + USED AGAINST SERUM TESTED	NUMBER OF UNITS PER C.C. TESTED FOR		
	For	Result		300	325	350
September 2920 c.c. .21	-2½ ± -1½	.2	-1½	-1½ ±	-1½ ±

TABLE 5.
EHRlich's METHOD.

DATES	L+ TESTS WITH EHR- LICH'S SERUM		L+ USED AGAINST SERUM TESTED	NUMBER OF UNITS PER C.C. TESTED FOR					
	For	Result		250	300	312.5	325	337.5	350
May 1874 .745 .75	-4 -4 -3							
May 2774 .745	+ -5							
June 474	-6							
June 27745 .745	-8* -6	.745*	+	+				
July 674 .74	-8 -5	.74				-8		-3
July 15745	+	.745		+	+	+		
July 26745 .75	+	.75				+	-3	-3
August 2574 .745 .75	+	.745		+		-3	-3	
September 27745 .745	-3 -8				+	+		

*The tests for L+ when performed upon the same date as those upon the serum were actually a part of the same series, and the diluted toxin injected was taken from the same cylinders with the same pipette for both the L+ tests and the serum tests, thus controlling the dilution for the L+ with each serum test.

The member making the tests in Table 5 desires to call attention to the fact that when the controls of the L+ dose showed this to be too low, the serum tests showed a corresponding increase in the antitoxic power of the latter. (Tests on July 15 and 26). The errors in these tests must therefore have been in the making of the original toxin dilution if the previous and subsequent tests for the L+ and the antitoxin are to be taken as a standard. On those occasions an unusual number of tests made a slight change of technique necessary.

The results of the tests shown in the various tables may be summarized as follows:

Taking the end reaction in Table 1 as the neutralization of the toxin, as prescribed for that method, the results indicate that the serum contained 300-325 units of antitoxin per c.c.

Taking the end reaction in the tests performed according to the Ehrlich method, as the death of the guinea pig on the fourth day, the results indicate as follows:

Table 2.	310-320	units per c.c.
" 3.	275-287.5	" " "
" 4.	Less than 300	" " "
" 5.	312.5-325	" " "

While these results appear to show a fair degree of uniformity for a series of such tests, an examination of the tables shows that many individual results were quite out of harmony with the average, especially when slight changes in technique occurred. This would be more noticeable if all the tests performed on the serum were included, as was not the case in Tables 5 and 2, where some of the tests for L + were not given.

In the interests of economy of effort and in order to limit the number of tests which need to be performed on any one serum, and yet to attain as great an approach to accuracy and uniformity in results as possible, it has been deemed best by your committee to recommend that the procedures and precautions outlined below be adhered to in the making of tests for the determination of the strength of diphtheria antitoxic sera.

The committee recommend the adoption of the method for such procedures devised by Ehrlich, which, in a general way may be outlined as follows: First, the determination of the amount of diphtheria toxin necessary to kill in four days a guinea pig of approximately 250 grams weight when mixed with one unit of a Standard Antitoxin; second, the determination of that amount of a serum which when mixed with this dose of toxin will prevent the death of a guinea pig of the same weight for four full days. This amount of a serum is to be considered as containing one unit of diphtheria antitoxin.

For the making of dilutions of both toxin or antitoxic sera, including the Standard Serum, the following recommendations are made:

First, that a sterile, .85 per cent solution of sodium chloride (C.P.) in water be used as the diluting medium.

Second, that either sterile glass containers (measuring cylinders and flasks), accurately graduated to contain the desired amount of salt solution or of dilutions be used, or that sterile ungraduated containers to which the salt solution is distributed from accurately

graduated burettes be substituted. Preferably the burettes should be those whose readings have been certified to by either the Prussian or American official testing bureau. (Prussian, *Physikalische technische Reichsanstalt*. American, Bureau of Standards, Washington).

Third, that for the measurement of the undiluted toxin or serum, including the standard serum sterile capacity bulb pipettes with one mark certified graduations on the stem be used. Such pipettes should be washed out in the solution receiving their contents.

Fourth, that for the measurement of the diluted toxin or serum, sterile bulb outflow pipettes with certified graduations upon the stem be used.

Fifth, that the toxins and serums used be removed from the refrigerator just before their measurement, and that the salt solution be at room temperature.

Sixth, that the total amount of the mixture of toxin and serum dilutions to be injected be as closely approximate to four c.c. as possible.

Concerning the size and number of the dilutions of both toxin and serum, the committee have decided not to recommend any one scheme, believing that on any plan rigidly adhered to, and with the precautions recommended strictly observed, any suitable method should give approximately uniform results.

Several plans are used by the various members of the committee, and an outline of some of these will appear as an appendix to the report.

In injecting the mixture of toxin and antitoxin the following precautions are recommended:

First, that the syringe employed be one that will uniformly deliver its entire contents, including the emptying of the needle. For this purpose it has been found that the syringes of the Koch type are to be preferred to those having pistons. The system worked out by Rosenau¹ of using the barrels of specially devised syringes of the Koch type as mixing chambers for the toxin and serum dilutions fulfils the above requirement, and has the advantage of

¹ *Treas. Dept., Pub. Health and Mar. Hosp. Serv., Hyg. Lab. Bull. No. 19, October, 1904.*

doing away with the extra mixing chamber, and the necessity of drawing the mixture up into a syringe from such a chamber.

Second, that the mixture of toxin and serum dilutions be thorough and complete. To that end, and to permit time for at least the beginning of the union of toxin and antitoxin to take place in vitro, it is recommended that the mixture stand for 15 minutes at room temperature (when not below 15° C.) before injection.

Third, that the injection be made subcutaneously into the subcutaneous tissues of the anterior abdominal wall of the selected guinea pig, the needle being introduced posteriorly and directed toward the median line.

Fourth, that the hair of the animal over the site of penetration be removed before the operation.

In the selection and general treatment of the guinea pigs to be the subject of these tests care should be exercised.

One of the members of the committee¹ has called special attention to the existence of strains which are unusually resistant to the poisonous effects of diphtheria toxin. This insusceptibility is transferred from the female possessing it to her offspring.

In laboratories raising their own guinea pigs care should be exercised in using for breeding purposes females which have survived injections of either toxin or toxin-antitoxin mixtures, inasmuch as their survival may have been due to a special resistance to diphtheria toxin, and this character may be transmitted to their offspring.

The following recommendations are made concerning the selection and treatment of guinea pigs:

First, that only half-grown animals in excellent condition, and born of mothers not known to be unusually resistant to diphtheria toxin be used for these tests.

Second, that the animals shall weigh before feeding on the morning of the day of operation not under 235 or over 275 grammes. Experience has shown that the animals coming within these limits are for all practical purposes evenly susceptible to diphtheria toxin.

¹THEOBALD SMITH. *Jour. of Med. Research*, 1905, 13, pp. 341, 348.

Third, that after operation the animals shall be kept in cages allowing three-quarters to one square foot of floor space per animal, without handling or disturbance other than what is absolutely necessary for careful daily weighing* and for feeding them, for four full days. During this time any evidences of a pathological condition which can be observed without disturbance should be recorded.

Fourth, that after this period of four days the animal should be examined and weighed, and any condition of edema, induration, or necrosis at the site of injection recorded.

Fifth, that if the animal dies as a result of the test an autopsy should be performed, and the macroscopic pathological conditions noted. Especial attention should be paid to ascertaining whether the injection had been properly made into the subcutaneous tissues or had been accidentally made into the abdominal cavity.

Your committee believe that an observance of the recommendations herein made will strongly tend towards uniformity of results and ease in the application of the test.

Inasmuch as the committee were without power to add to their number, they desire to state that Dr. M. J. Rosenau, Director of the Hygienic Laboratory, United States Public Health and Marine Hospital Service, was invited to take part in their deliberations and that they are indebted to him for valuable suggestions.

Respectfully submitted,

HERBERT D. PEASE, *Chairman.*

J. J. KINYOUN.

JOSEPH MCFARLAND.

WM. H. PARK.

THEOBALD SMITH.

APPENDIX.

The choice of a system of diluting both the toxins and the sera in the determination of L+ dose, and in the regular tests for antitoxic strength, will depend on one's choice in the method of making the final injection of the mixtures. The methods in vogue for the latter are of two types: first, those that aim so to arrange the systems of dilution as to give approximately two

* Any handling of animals after injection may affect them injuriously and some members do not recommend the daily weighing. Any course decided upon should be adhered to.

c.c. each of the toxin and serum dilutions, and secondly, those that make up the final total of four c.c. by the addition of salt solution either to the final mixture or by using the additional salt solution for washing the last slight traces of the mixture in the syringe into the guinea pig.

If the first plan is chosen, then it is necessary to arrange the dilutions of the toxin so that the dose to be injected will be about two c.c. For example, if the L+ of a given toxin was suspected, or had been determined to be .21 c.c., it would require a dilution of 10 times in order to give that amount of toxin in approximately two c.c. (actually 2.1 c.c.) of the dilution.

For the dilution of the Standard Serum, the dilution given on the bottle as necessary to obtain one unit in one c.c. would have to be doubled in order to obtain one unit in two c.c. For example, where the bottle label gives one c.c. of serum mixture plus 12.25 c.c. of diluent as a dilution containing one unit per c.c., it would be necessary to make a dilution of one c.c. of serum plus 25.5 c.c. of diluent in order to obtain one unit in two c.c.

For the dilution of the sera to be tested, all that is necessary under this system is to make dilutions such that the denominator of the fraction, which represents the amount of serum, in two c.c. of dilution, is the same as the number of units per c.c. suspected of being contained in the serum to be tested.

Thus, if a given serum is to be tested for a possible strength of 300 units per c.c., a dilution of 1 c.c. serum + 99 c.c. salt solution, and of this 2 c.c. + 10 c.c. of salt solution will give $\frac{1}{100}$ c.c. of the original serum in 2 c.c. of dilution. By this system the addition or subtraction of 1 c.c. to or from the 10 c.c. of salt solution in the second dilution will raise or lower the units to be tested for by 25.

Where the other type of system is used, any or even no dilution of the toxin, and any dilution of the serum, giving in the amount injected that fraction of one c.c. of serum, the denominator of which represents the number of units to be tested for, will be sufficient for the performance of the test, and will only require the addition of the salt solution to make the total mixture four c.c. For example:

Toxin L+ = .21 .21 measured directly by pipette into cylinder holding final mixture or diluted 1 c.c. toxin + 4 c.c. salt sol. = .21 c.c. in 1.05 c.c. dilution.

Serum to be tested for 300 units per c.c.:

1 c.c. serum + 19 c.c. salt sol.

1 c.c. of first dilution + 14 c.c. salt sol. = $\frac{1}{100}$ c.c. in 1 c.c. of second dilution.

Or another method:

1 c.c. serum + 9 c.c. salt sol.

1 c.c. of first dilution + 29 c.c. salt sol. = $\frac{1}{100}$ c.c. in 1 c.c. of second dilution.

The opportunities for error in measurement are, of course, the least in the greater dilution.

THE IMPORTANCE OF THE PARADYSENTERY BACILLI.

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THE investigations upon the bacteriology of dysentery in the United States have revealed the fact that in many of the sporadic cases and in quite a few epidemics none of the bacilli described by Shiga have been present. In cases occurring in the Middle Atlantic and New England states there have been found chiefly two types of bacilli which though resembling the Shiga bacilli in many respects yet differ from them in others of great importance, viz.: in specific agglutinins and immune bodies and in the formation of indol and in the fermentation of mannite in peptone solution. In this medium one type also actively ferments maltose and saccharose, while the other type does so very feebly, if at all. In a few sporadic cases, several different varieties of bacilli varying from these types have been the only organisms found. The prevalence of the cases due to these bacilli is underestimated, since many of them have been reported, as if due to the Shiga bacillus. This is because the cultures were not at first carefully studied, or because the name of Shiga was given out of compliment to the one who, in establishing the bacillary origin of dysentery in temperate climates, opened the way to the discovery of the bacilli differing from the type discovered by him. Few realize that the great majority of the cases of dysentery in Baltimore, as well as those in New York, Boston and Philadelphia have not revealed the Shiga bacillus, but have revealed the bacilli of the types designated by us as the paradysentery bacilli.

It seems to us that the statement of Hiss,¹ that further investigation is necessary to establish the claim that these paradysentery bacilli are inciters of disease is too conservative. In the article by Drs. Collins,² Goodwin, and myself we collected a number of sporadic and epidemic cases of dysentery in which

¹ *Jour. of Med. Research*, 1904, 13, p. 40.

² *Ibid.*, 11, p. 553.

paradysentery bacilli were the only organisms of the dysentery type present.

These bacilli were present alone in so many cases that the proof that they were inciters of the disease seemed as conclusively shown as in the cases where the Shiga bacillus was found.

During the summer of 1904 we investigated a number of cases of dysentery in New York City and they gave the same findings as those of the years previously reported. Only a small percentage of the cases contained Shiga bacilli, all the others containing one or another of the paradysentery varieties. On Staten Island, which is situated about six miles from New York City, in the bay there was a severe localized epidemic of dysentery, from which all the cases examined gave the Shiga bacillus.

An epidemic at a dumping station was so striking in that the people were isolated from those outside that, although previously published, it is here repeated.

At Rikers' Island a number of men were filling in new land. The privy arrangements were very poor, and infection readily took place. Dysentery broke out and spread to a number of the men, as well as to the physician in charge. Those infected had usually a short, sharp attack with a quick recovery. Very large amounts of blood were passed by some of the sick. In some a large proportion of the bacteria isolated were bacilli of the Manila type. (Flexner). No other type of dysentery bacilli was found in any of the cases in this epidemic.

One of last summer's cases gave very interesting findings as seen in the following account:

Girl, 8 years. Had an acute attack of dysentery with tenesmus, abdominal pain and frequent passages. Pronounced symptoms lasted eight days. On the third day a stool was put into four ounces of sterile water and sent directly to the laboratory. When received there was a little blood and a moderate amount of mucus present with the fecal matter. A series of plates was made by Dr. Mary E. Goodwin from a piece of bloody mucus. 69 colonies were fished from them at the end of 20 hours. Of these 69 colonies 50 were formed of bacilli of one of the mannite fermenting paradysentery types. The organism isolated agglutinated as follows in the patient's blood:

	1-100	1-200	1-400
Organism isolated	++	±	—
Paradysentery b. Mt. Desert type (Park)	+++	+	—
Paradysentery b. Manila type (Flexner)	+	—	—
Dysentery b. (Shiga)	—	—	—

The agglutination of the Manila type in 1:100 dilutions was due to common agglutinins and is of almost constant occurrence in the blood of those suffering from colon or dysentery bacillus infections. When the culture was placed in a serum which after purification by extraction of agglutinins contained specific agglutinins for the Manila type only no agglutination took place at any dilution, while in a serum containing specific agglutinins for the Mt. Desert type it agglutinated completely, but, as in the case of the patient's serum in slightly lower dilutions than the laboratory culture of the Mt. Desert type, which had been kept on agar for three years.

In this case where the examinations were made by Dr. Goodwin we have characteristic symptoms, the presence during the disease of abundant bacilli of a single paradysentery type and no bacilli of any other dysentery or paradysentery type, the development of specific agglutinins for that type and for no other and the disappearance of the bacilli with the cessation of the symptoms.

The causal relationship between this bacillus and the case is to my mind as well proven as in any case of dysentery in which the Shiga bacillus was found in the bloody mucus and specific agglutinins in the blood.

The mannite fermenting types are widely scattered over the world, and to me they seem to be inciters of characteristic cases and epidemics of dysentery, although on the average the disease caused by them is milder than when due to the Shiga bacillus. One or the other of these types also appear at times in small numbers in mixed infections where dysenteric symptoms are almost or entirely absent.

It seems to me convenient and proper to restrict the name dysentery to bacilli having the characteristics of the bacillus first identified by Shiga, and give the name paradysentery to the other varieties of bacilli which approach more closely the colon group in that they produce indol and have a greater range of activity in fermenting carbohydrates. While it seems wise to separate the dysentery and paradysentery bacilli, the name dysentery remains a purely clinical term and includes under it cases excited by both varieties of bacilli as well as by protozoa.

THE OCCURRENCE OF BACTERIUM PNEUMONIAE IN THE SALIVA OF HEALTHY INDIVIDUALS.*

W. D. FROST, C. B. DIVINE AND C. W. REINEKING.

(From the Bacteriological Laboratories of the University of Wisconsin.)

INTRODUCTION.

It is generally recognized that *Bacterium pneumoniae*† is found in the saliva of a considerable proportion of healthy individuals. The statement is frequently made that it occurs in one out of every five persons. But the experiments upon which these conclusions are based have been made by different observers at various times of the year and under a variety of conditions; so much so that it seemed worth while to restudy this question and to determine independently the distribution of this germ in the saliva. The salivas of some 50 individuals have been examined, and in some cases that of the same individuals at different times of the year. This was done to determine whether or not there was any variation in the seasonal distribution of the germ. The results seem to show that there is marked variation in the distribution of this germ at different seasons of the year. Other examinations have been made upon different classes of individuals at the same season of the year to note if any variation occurred which could be properly assigned to differences of occupation. Here again a variation appeared, especially when the distribution of the germ in the saliva of those employed "indoors" was compared with the distribution of the germ in the saliva of those employed "out of doors." The authors are well aware that the conclusions which might be drawn from these experiments are not conclusive, but it is believed that the results obtained are of sufficient import to warrant a somewhat detailed account of the experiments and the data collected.

*The work here described formed the basis for theses which were presented for the bachelor's degree at the University of Wisconsin, by Mr. (now Dr.) Divine in 1900 and by Mr. Reineking in 1904.

†Throughout this paper the term *Bacterium pneumoniae* is used instead of the more common term of "pneumococcus." This is in accordance with Migula's system of classification, which seems to be the one most widely accepted.

METHODS EMPLOYED.

The presence of *Bacterium pneumoniae* has been determined exclusively by the inoculation of rabbits with the saliva. The inoculations have been made as soon as possible after collection. The rabbits received approximately two c.c. intraperitoneally. In cases where the animals died the autopsies were performed as soon as possible. Cover-slips of the blood from the various organs were stained by means of the Welch capsule stain. Cultures were also made from the various organs. At first these were made on blood agar prepared according to the method recommended by Eyre and Washburn.¹ In this case the identification of *Bacterium pneumoniae* was confirmed by the appearance on this agar of small pinhead colonies. Later cultures were made directly into milk. In this medium the organism in question produces a thick curd and cover-slip preparations therefrom show beautiful capsules with the capsule stain. Those cases only were counted as positive in which there developed a septicemia and in which there was found in the blood of the rabbit, or milk subcultures therefrom, a lancet shaped diplobacillus and on which a definitely stained capsule could be demonstrated. In a few cases in order to economize on rabbits the saliva of several individuals, which seemed unlikely to contain the germ, were mixed and the mixture inoculated into a single rabbit. In case the rabbit died it was necessary to retest the saliva of each individual separately; but if the animal remained well it was taken for granted that none of the group contained the organism in question.

EXPERIMENTAL WORK.

From Table 1, which gives the details of the work, it is seen that there have been 85 inoculations. Thirty-two of these gave a positive result, or 37.6 per cent. The saliva was obtained from 50 different individuals. Eighteen of these showed the presence of this germ, or 36 per cent. The percentage of positive results obtained is higher than that reported by previous observers.

Of the 50 individuals tested 26 were university students, 2 were high school children, 6 were ward school children, 5 were teamsters, 5 were outside laborers (carpenters), 5 were draftsmen and 1 was a housewife. All were well at the time the saliva was collected and it is not known that any of them fell sick for some-time afterward, so that they may fairly be considered to have been healthy at the time their saliva was examined.

SEASONAL DISTRIBUTION.

A considerable part of the inoculations were undertaken, as already indicated, for the purpose of determining whether or not

Jour. of Path. and Bact., 1908, 5, p. 13.

TABLE 1.
INOCULATION OF RABBITS WITH THE SALIVA OF HEALTHY INDIVIDUALS.

NO. OF EXPERIMENT	INDIVIDUAL	TIME OF INOCULATION	RESULT		NO. OF EXPERIMENT	INDIVIDUAL	TIME OF INOCULATION	RESULT	
			Neg.	Pos.				Neg.	Pos.
1.....	A1	Oct.-Nov.	0		44.....	C5	Jan.-Feb.	0	
2.....	A2	"	0		45.....	D1	"		+
3.....	A3	"		+	46.....	D2	"	0	
4.....	A4	"	0		47.....	D3	"	0	
5A.....	A5	"	0		48.....	E1	"	0	
6.....	B1	"	0		49.....	E2	"		+
7.....	B2	"		+	50.....	E3	"	0	
8.....	B3	"	0		51.....	E4	"	0	
9.....	B4	"		+	52.....	E5	"		+
10.....	C1	"	0		53.....	F1	"	0	
11.....	C2	"	0		54.....	F2	"	0	
12.....	C3	"	0		55.....	F3	"	0	
13.....	C4	"	0		56.....	F4	"	0	
14.....	C5	"	0		57.....	G1	"		+
15.....	D1	"	0		58.....	G2	"	0	
16.....	D2	"	0		59.....	G3	"		+
17.....	D3	"	0		60.....	G4	"		+
18.....	E1	"	0		61.....	H1	"		+
19.....	E2	"	0		62.....	H2	"		+
20.....	E3	"	0		63.....	H3	"		+
21.....	E4	"	0		64.....	H4	"	0	
22.....	E5	"	0		65.....	H5	"	0	
23.....	F1	"	0		66.....	I1	Mch.-Apr.		+
24.....	F2	"	0		67.....	I2	"	0	
25.....	F3	"		+	68.....	I3	"	0	
26.....	F4	"		+	69.....	I4	"		+
27.....	G1	"		+	70.....	I5	"	0	
28.....	G2	"	0		71.....	J1	"	0	
29.....	G3	"		+	72.....	J2	"		+
30.....	G4	"	0		73.....	J3	"		+
31.....	A1	Jan.-Feb.		+	74.....	J4	"	0	
32.....	A2	"		+	75.....	J5	"		+
33.....	A3	"		+	76.....	K1	"	0	
34.....	A4	"	0		77.....	K2	"	0	
35.....	A5	"	0		78.....	K3	"	0	
36.....	B1	"		+	79.....	K4	"	0	
37.....	B2	"	0		80.....	K5	"	0	
38.....	B3	"		+	81.....	L1	"		+
39.....	B4	"		+	82.....	L2	"		+
40.....	C1	"	0		83.....	L3	"		+
41.....	C2	"	0		84.....	L4	"		+
42.....	C3	"	0		85.....	L5	"		+
43.....	C4	"	0						

the season of the year had any influence on the prevalence of the germ in the saliva. On account of the large number of rabbits required periods were selected about two months apart. In the year 1900 the saliva of 30 individuals was examined in the fall and then again in the winter months. The figures given for the spring months were obtained in 1904 and from an entirely different set of people. While the results obtained then in the first and second periods are directly comparable, the results obtained for the spring months can not be so closely compared with the preceding. The results obtained show that of the 30 examina-

tions made in the fall 7 were positive, or approximately 24 per cent; that of the 35 inoculations made in the winter 15, or 43 per cent, were positive; and that of the 20 inoculations in the spring 10, or 50 per cent, were positive.

VIRULENCE OF THE GERM.

Another question of considerable importance is that of the virulence of the germ of pneumonia as it occurs in the saliva. This has been judged entirely by the period elapsing between the time of inoculation and the death of the rabbits. This time has varied from 20 to 100 hours. The details are represented graphically in Fig. 1.

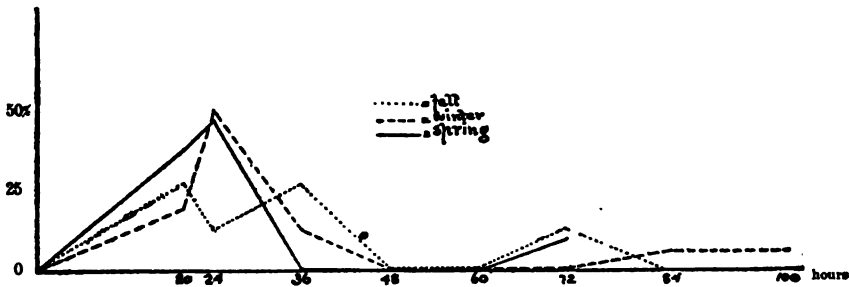


FIG. 1.—Virulence of *Bacterium pneumoniae* as indicated by the course of the disease in rabbits. The ordinates represent the per cent of rabbits which died at or within a given time. The abscissae are the intervals expressed in hours.

It would thus appear that the virulence of this germ was greater during the time when it was most prevalent and lower when it was less common. In other words it appears that 90 per cent of the rabbits died within 24 hours in the spring, about 73 per cent in the winter, and only about 42 per cent in the fall months.

PERSISTENCE OF THE GERM IN THE SALIVA.

Still another point of some interest is the question as to whether or not the germ persists in the same throat for sometime. The condition of affairs as it appeared in this work is shown in the following table:

TABLE 2.
PERSISTENCE OF THE PNEUMONIA GERM IN THE SAME THROAT AT DIFFERENT SEASONS
OF THE YEAR.

	GROUP														
	A1	A2	A3	B1	B2	B3	B4	D1	E2	E3	F3	F4	G1	G3	G4
Fall	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Winter	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

It is seen here that of the 7 individuals whose saliva contained *Bacterium pneumoniae* in the fall only 4 contained it in the winter months, and that of the 12 individuals who harbored it in the winter 8 had acquired it since the previous examination. Or again only 4 out of the 15 gave the same reaction at both examinations.

INFLUENCE OF OCCUPATION.

In regard to the influence of occupation on the presence of this germ in saliva but little can be determined from the earlier experiments (Nos. 1 to 60) since they were not performed with this idea in view and all of the subjects were selected from practically the same occupation; but the latter experiments (Nos. 61 to 85) bear directly on this subject. In this part of the work three classes were studied: those who were indoors almost entirely, those in and out, and those who worked outside all day. For convenience they were divided into groups. Group L consisted of five draftsmen, working in a machine shop. Groups H and I were five university students. The group designated J were teamsters or hack drivers who worked outside a great deal but not constantly. The last group (K) was composed of five laborers who worked out of doors all day all of the year around as carpenters.

TABLE 3.
INFLUENCE OF OCCUPATION ON THE DISTRIBUTION OF THE PNEUMONIA GERM IN SALIVA.

Group	No. of Indi- viduals Tested	Occupation	No. of Indi- viduals Reacting	Per Cent Reacting
K	5	Laborers	0	0
J	5	Teamsters	2	40
I	5	Students	2	40
H	5	Students	3	60
L	5	Draftsmen	5	100

These results suggest that occupation may be a factor in accounting for the variation of the distribution of this germ in the saliva of healthy individuals.

SUMMARY.

Eighty-five rabbits have been inoculated with saliva causing sputum septicemia in 32 or 37.6 per cent.

The saliva of 50 different individuals has been examined and in 18 or 36 per cent of the cases *Bacterium pneumoniae* has been found.

The frequency with which this germ appears seems to vary with the season of the year, 23 per cent of the salivas examined in the fall being positive, 43 per cent in the winter and 50 per cent in the spring.

The virulence* of the germ, as indicated by the rapidity of the course of the disease in the rabbit, varies, the greatest virulence appearing during the period of greatest frequency.

The germ may persist in the saliva of certain individuals from season to season but more than 66 per cent of the cases positive in winter were negative in the fall.

Bacterium pneumoniae appears to be more frequent in the saliva of individuals working "indoors" than those working "outdoors."

*No account is taken of the number of germs that might be present.

LABORATORY METHODS AND DEVICES.

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(From the Minnesota State Board of Health Laboratory and the Department of Pathology and Bacteriology, University of Minnesota.)*

APPARATUS FOR THE COUNTING OF COLONIES IN PETRI DISHES.

(Designed March, 1904, and Described by Louis Blanchard Wilson.)

The apparatus consists essentially of a dish holder bearing a circular glass plate appropriately ruled with circular and radiating lines. By means of a three-jawed chuck acting like an iris diaphragm, the Petri dish by a single motion of a lever is accurately centered over the ruled plate and securely held in place. In this position colonies in cultures which for any reason may not be inverted (e. g. much liquefied gelatin cultures) are counted. But preparations which may be inverted (e. g. all agar cultures and all gelatin cultures only slightly liquefied) are readily turned over by rotating the holder, thus bringing the ruled plate above the bottom of the inverted dish and placing the preparation in the most advantageous position for counting.

Under the dish holder is placed an adjustable swinging plane mirror of larger diameter than the dish. The obverse side of the mirror is dead black.

Above the holder is a swinging arm for holding a lens of any desired (low) magnification. The lens, by means of joints and a revolving sleeve, may be focused readily on any portion of the culture. (The designer prefers a large, low-power $2\frac{1}{2}$ diameters "reading glass" for routine work.)

The above parts are supported on a heavy rod rising from an ample horseshoe base. With the exception of the glass portions the apparatus is constructed entirely of brass, polished and lacquered, and is thus durable and neat. It is made by Mr.

*Some of these methods were presented before the Laboratory Section of the American Public Health Association at Buffalo in 1901, but no published description appeared from either of the laboratories. They were therefore included in the demonstration before the Section at the Havana meeting, 1905, and embody suggestions and devices originating with different members of the two staffs and are here presented by the Director of the laboratories.

L. U. Boyle, Mechanician, Medical Laboratories, University of Minnesota, who also has solved most of the mechanical problems involved, though valuable suggestions have been received from Drs. M. Russell Wilcox and E. H. Beckman.

A photograph of the apparatus is shown in Fig. 1.

The advantages of the apparatus are as follows:

1. The Petri dish culture may be instantly changed from the erect to the inverted position or to any inclined angle without changing the relative position of the ruled glass plate, the ruled side of which is held constantly in apposition to the bottom of the dish.

2. The dish is always accurately centered over the ruled plate. This greatly diminishes the error in estimates made from alternate counted segments of preparations in which the colonies are too numerous to permit of all being counted.

3. Every advantage of light is afforded by the large swinging mirror with its reflecting and dead-black surfaces.

4. The lens holder is readily adjustable and permits the use of various lenses, to which may be attached a *camera lucida*.



FIG. 1.

STANDS FOR MUSEUM EXHIBITION OF PERMANENT FIXED CULTURES OF BACTERIA.

These stands are constructed and the parts assembled by Mr. L. U. Boyle. The stands themselves, the component parts, and the methods are illustrated by the accompanying photograph (Fig. 2). The wooden frame is made of closely grained wood, suitably stained, and consists of a heavy doubly beveled base with two pillars, across the top of which is a flat support, perforated. The cultures are arrested at a suitable stage of their

development by fixation with formalin. The cotton plugs are withdrawn from the mouths of ordinary test tubes in which the cultures are grown. The test tubes are placed in large airtight jars and formalin poured into the bottom of the jar or upon cotton. The formation of formic acid may be prevented by the addition of ordinary chalk. For gelatin stab cultures or deep

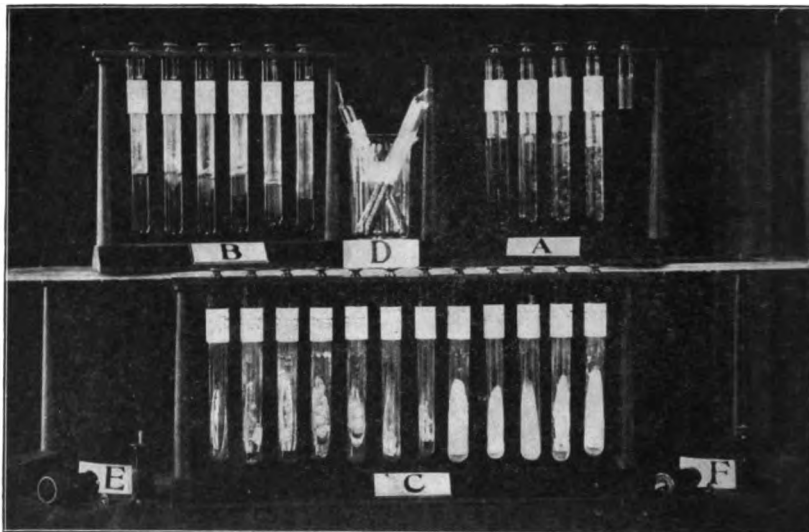


FIG. 2.

cultures, such as anaërobes, in which it is imperative to stop immediately the growth of the culture in the depth of the medium, the formalin may be added by a small glass pipette blown from ordinary glass tubing. The pipette filled with formalin is thrust to the bottom of the culture tube along one side, and as it is withdrawn the formalin is deposited in the pipette track.

When properly fixed, the test tubes are sealed in the blow-pipe flame, and in such a way as to leave a knob or a hook on the top. The hermetical sealing prevents evaporation (D, Fig. 2). The caps for these tubes are made from ordinary gun shells, which can be obtained of various gauges. Those having high brass bases are preferable. The shells are cut off and the cap

extracted. The percussion cap is replaced by a threaded brass pin, driven firmly into position. The nuts are made with milled heads. The preserved, sealed cultures are fixed in the cartridges with moist plaster of Paris. The threaded stem is passed through the perforation in the wooden support and the nut screws the tube in position. Labels may be attached to both the tubes and the stand and coated either with shellac or paraffin.

The special advantages of this method are,

1. The entire tube and its contents are shown. The ordinary test tube rack obscures the bottom of the tube.
2. Facility in grouping the cultures is afforded.
3. The permanency of all cultures, except chromogens which are affected by the formalin, is assured, since evaporation is prevented.

COMPLETE AND RAPID ANAEROBIOSIS.

Fig. 3 illustrates the combination of methods which is employed. A Kipp's hydrogen generator and two wash bottles for cleaning the gas are placed on a shelf in the neighborhood of a sink. A Lautenschläger or other suitable modification of the Bunsen pump, is placed in position over a sink and brass or other metal pipe connects the water pump with a mercury or other manometer and is finally connected with the Kipp's apparatus by means of a Y tube. The third limb of the Y is connected with a jar or sectional receptacle designed by Novy.

For the inoculation of agar, gelatin or broth tubes, the medium is boiled immediately before use and inoculated with the microorganisms which it is desired to observe either after the medium has been set or, in the case of "shake" cultures, before solidification occurs. A wide-mouthed bottle has a layer of sand in the bottom. A solution of pyrogallic acid is poured upon the sand. The tubes are placed in position in the bottle. The wide-mouthed bottle is provided with a rubber stopper having one perforation. In the perforation a bent glass tube with a ground gas-tight, freshly vaselined cock, is fitted. The rubber stopper is coated with paraffin so as to make a gas-tight joint with the neck of the bottle and to prevent the adherence of the rubber to the glass. Just prior to the insertion of the stopper into the

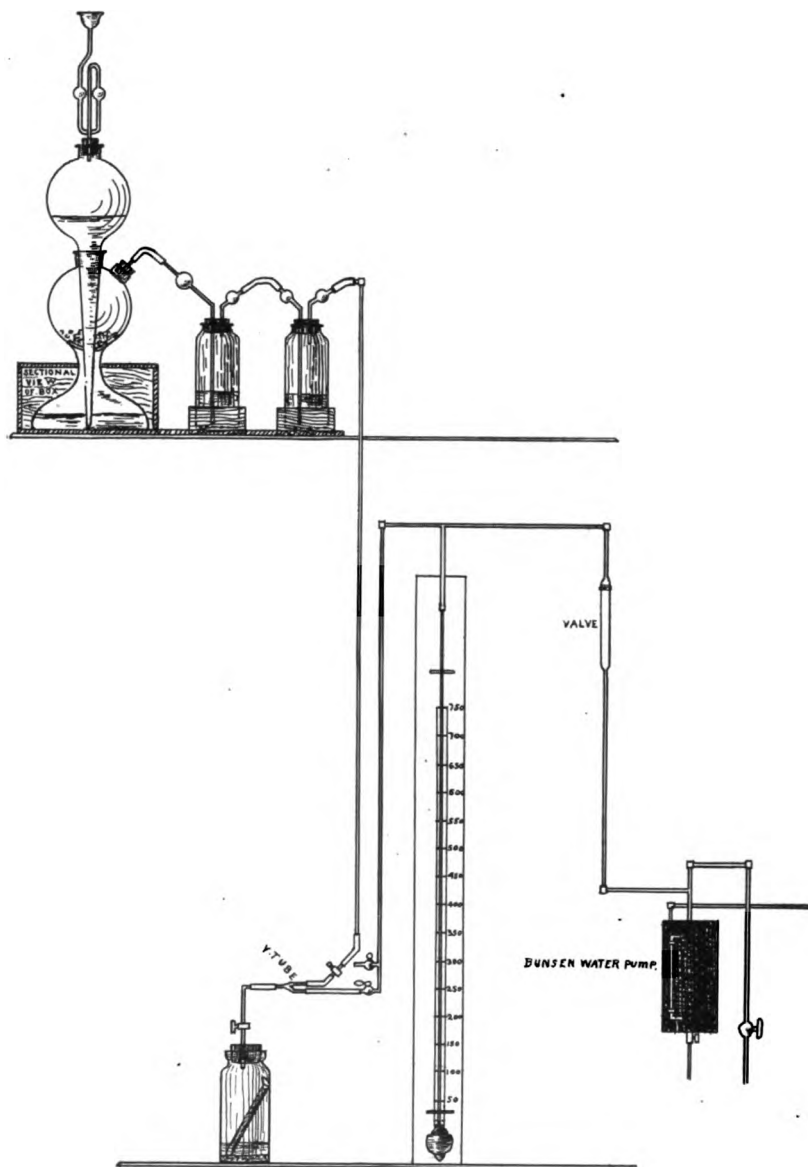


FIG. 3.

bottle a small stick of caustic soda or potash is dropped into the pyrogallic solution. The water pump is used for exhausting the air in the bottles and the cock in the tube leading from the Kipp's apparatus is turned off, both cocks between the pump and the bottle being turned on. When exhaustion is complete, the tap between the Y tube and the pump is turned off and the tap on the tube leading from the Kipp's apparatus turned on very gradually in order that hydrogen may replace the air which has been exhausted from the bottle. Care should be taken not to permit the hydrogen to escape so fast that air is drawn in through the top of the Kipp's apparatus. When the bottle is full of hydrogen, the tap leading from the hydrogen generator is closed and the bottle is again exhausted of its mixture of air and hydrogen. When this is complete, the tap leading to the pump is again closed and more hydrogen admitted. This process is repeated five or six times or oftener, if desired, until all of the air has been completely washed from the bottle, when suction should be applied until a negative pressure of 100 to 150 millimeters of mercury is recorded. The cock in the bent tube in the rubber stopper is now closed and the bottle disconnected from the Y tube. With its contained cultures the bottle is placed in the incubator. The reason for the negative pressure is that it obviates the danger of the expulsion of the stopper when the cool bottle and its contents are placed in the incubator and the contained gas expands. For plates the large sectional receptacle designed by Novy for anaërobic cultures may be used in the same way by replacing the complicated glass stopper with a perforated rubber stopper with bent glass tube and stop cock.

The special advantages of the apparatus and method are the following:

1. No table space is permanently occupied.
2. If gas-tight joints and cocks are provided the apparatus is always ready for use and it is almost as simple to grow anaërobic as aërobic.
3. By firmly fixing the Kipp's apparatus and the two wash bottles to a board the whole apparatus may be taken down very easily for cleaning.

4. If exhaustion of the air follows immediately after the addition of the sodium hydrate the fluid in the bottle remains colorless and this lack of color together with the maintenance of a slight negative pressure, may be taken as the index of complete anaërobiosis.

5. There is a saving of hydrogen as compared with Botkin's or Novy's method.

6. The complete anaërobiosis secured is desirable in testing unknown microorganisms and in the elimination of aërobes during the isolation and identification of bacteria in mixtures.

7. The use of complicated, expensive and fragile apparatus is rendered unnecessary.

BI-METALLIC THERMO-REGULATOR WITH SPECIAL ARRANGEMENT FOR EASY CLEANING AND ADJUSTMENT.

Mr. L. U. Boyle, the laboratory mechanician, is the designer and maker of a thermo-regulator which is very like the French pattern. Without a series of complicated sketches or photographs it would be impossible to illustrate it.

The main features are the provision of small metal union joints whereby it is possible to attach a lead or other metal tube directly to the gas supply, to the thermo-regulator, and from the thermo-regulator to the safety lamp. These unions provide a means of easily disconnecting the thermo-regulator or the safety burner from its metal connections. Metal connections for incubators are universally employed throughout these laboratories as being much safer than rubber. Bi-metallic thermo-regulators, with the special union joints, render manipulations as easy as with rubber tubes and protect against the danger of fire.

DEVICES FOR DISPLAYING AND LABELING PATHOLOGICAL SPECIMENS.

Fig. 4 shows several features which have been found of great practical use in the pathological museum:

1. Labeling specimens in Whitall-Tatum jars. A satisfactory method of labeling was found to be difficult without obscuring the specimens. The objection to the attachment of the label holder or label to the clamp or top of the jar is that it is apt

to become detached from the specimen or jar to which it rightfully belongs. Thin sheet brass or copper is obtained already cut to size. Along one side and both ends the metal is bent so as to form a groove into which a label card slips. In the center of the

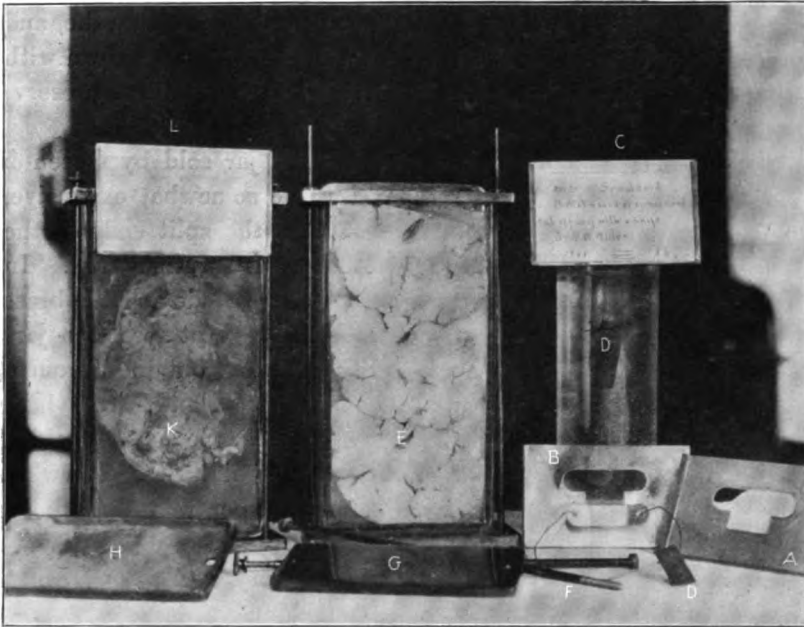


FIG. 4.

sheet, by means of a punch, an opening which is somewhat T-shaped is made so that the metal is severed except at the base. This metal is bent backwards, first at right angles to the plane of the sheet, then again at right angles so that what was formerly the top of the T is now in a plane parallel to the body of the label holder. Through each end of this a hole is punched and annealed copper or brass wire is passed through the holes. This is then applied to the neck of the jar. By means of pliers, or the fingers, the wire is tightly twisted so as to support the label holder in position. A shows the front view of the label holder.

B shows the back view with the copper wire in place. *C* shows the label holder containing the label in position.

2. In order to avoid the possible return of a specimen to the wrong jar when once it has been removed, all specimens when received in the laboratory are provided with an accession number. A small tag, *D*, made of stalbite or wood fiber, such as that used by electricians for insulating purposes, is numbered with a die, and through a hole which has been previously drilled is sewn with stout silk into the specimen before it is immersed in the preserving fluid.

3. Other specimen jars. The "Coplin" jar sold by Queen & Co. is shown in *E*. This was found to be somewhat expensive, and the method of fixation of the label in the split ends of the rods which project above the specimen jar was unsatisfactory. In the workshop of the laboratory, patterns were made and the brass rods and bases were cast in a local foundry. Flat specimen jars were imported and solid India rubber gaskets obtained from a local firm. *F* shows the rods, *G* shows the top, and *H* the base of the jar. *K* shows the jar which has been made and assembled in our laboratory workshop. *L* shows a label holder somewhat like that already described, but without the central punch hole, which has been soldered to the brass top. This method of preserving has been found to be very satisfactory, and a jar such as shown in *K* is more easily manipulated and cheaper than the "Coplin" jar, and does not require so much perpendicular space for display. It is unnecessary also to detach the label in opening the jar.

AGAR HANGING DROP.

In 1896 a method was devised which would render it possible to study the development of a bacillus from a spore, or vice versa, and which would permit of special observation on the arrangement of bacteria, i. e., their relation to each other.

A coverslip is placed in coverslip forceps and sterilized by passage through the flame. A tube of melted agar is allowed to cool to about 50° C. With a platinum inoculator a minute amount of the microorganism to be observed is streaked in the center of the sterilized coverslip, and by means of a loop a large drop of

the molten agar is placed immediately over the streak. The coverslip is inverted over a vaselined hollow ground slide, and the development of the microorganism, which is now on the top of the flat plane of agar immediately underlying the coverslip, may be followed with an oil immersion lens. This method has been employed for teaching and research purposes for nine years in these laboratories. As an aid to the identification of *B. anthracis* it has proved of great value on more than one occasion.

Hill, who was not aware of the existence of this method, described a somewhat similar procedure, "the hanging block," in 1902.¹

DEVICES FOR STAINING A LARGE SERIES OF MICROSCOPIC CULTURE PREPARATIONS ON COVERSLEIPS OR ON SLIDES.

Two methods have been employed for large series of diphtheria examinations in the State Board of Health routine diagnostic work, where it is necessary to examine and report upon from 50 to 250 specimens in a day.

1. Until within the past year coverslip preparations have been made and mounted with balsam. The tubes as they are received from various localities are numbered serially, and the data slips which accompany them are correspondingly numbered. They are then placed in tumblers numbered 0 to 9. After being incubated overnight, the tumblers are placed in regular sequence in a row. Two kinds of coverslip forceps are used and grouped in sets of 10. The individual forceps in each set are numbered 0 to 9. When Cornet coverslip forceps are used the numbers are struck upon the upper surface with a metal die. When Stewart coverslips forceps are used a small disc of copper numbered with a metal die is slipped into place and soldered in the loop in the middle of the upper surface. By the use of these numbered coverslip forceps, the relationship of the coverslip preparation to the corresponding tube can be maintained without danger of confusion until the specimen is finally mounted on a labeled slide.

2. Recently, staining upon the slide has been employed, and in some respects is more convenient, although not so cleanly, nor are the mounts so permanent.

¹ *Jour. Med. Research*, 1902, 2, p. 202.

Fig. 5 shows a device first suggested by Dr. L. B. Wilson and improved by Drs. Beckman and McDaniel. It is constructed of metal and does not bend appreciably when heated. The slides are numbered with a glass pencil, and the preparations as made are placed in regular sequence in the apparatus, which consists essentially of a tray without a bottom other than the two strips of metal placed on edge. After the preparations are dried, the tray is placed crosswise over a sink and an ordinary Bunsen burner held in the hand of the operator is used for flaming—the depth of the sink permitting of the application of the flame to the under surface of the slides. Löffler's methylene blue or other stain is poured upon the upper surface of the slides and, if desired, heat



FIG. 5.

may be applied either to the upper surface or from underneath. Distilled or tap water by means of a rubber tube with a glass tip is used for washing the microscopic preparations without disturbing them in the tray. The whole tray is picked up, allowed to drain somewhat, then placed between two folds of smooth filter paper and blotted on both sides. It is convenient to place the tray upon a piece of blotting paper which is more than double the width of the tray; the edge of the blotting paper is then folded over the top of the tray and the slides are firmly pressed and rubbed with the hands through the blotting paper. By inverting the tray, still wrapped in the paper, all the slides are turned out upon the paper and the tray removed. The paper is again folded in position so as to be on both sides of the slides. It is now reinverted so as to leave the slides in regular sequence face up. When completely dried they are ready for examination, and the sequence has been maintained.

"WIDAL" REACTION IN TYPHOID FEVER.

An envelope is furnished to physicians which contains the following named articles:

1. A piece of aluminum wire, No. 19 gauge, 7.5 cm. long, bent at one end into a loop 0.3 cm. in diameter.
2. A strip of aluminum foil, No. 40 gauge, 5 cm. square.
3. A data blank as follows:

MINNESOTA STATE BOARD OF HEALTH LABORATORY
(University of Minnesota), Minneapolis.

DATA TO ACCOMPANY SPECIMEN FOR TYPHOID FEVER EXAMINATION.

Date and hour of collection.....

Patient's Name.....Address.....

Physician's Name.....Address.....

Health Officer's Name.....Address.....

Has this case been reported upon before?.... If so, give previous case No.....

Patient's Age..... Sex..... Temperature.....

How long since disease commenced?.....

What is the supposed source of infection?.....

When, if ever before, has the patient had Typhoid Fever?.....

Remarks.....

.....

Physician's diagnosis.....Do you desire a telegraphic report?.....

The envelope has printed on it the following:

MINNESOTA STATE BOARD OF HEALTH LABORATORY
(University of Minnesota), Minneapolis.

OUTFIT FOR COLLECTING SPECIMEN OF BLOOD FOR SERUM DIAGNOSIS OF TYPHOID FEVER.

To secure a reliable reaction with dried blood it is necessary that a comparatively large amount be collected in as cleanly a manner as possible. Hence please observe carefully the following directions:

Wash with boiled water the part from which the blood is to be obtained (the lobe of the ear, end of finger, or toe in infant). Prick deeply the skin with a clean needle or scalpel. Remove four or five loopfuls of blood with the wire loop in outfit, placing each by itself near one edge of the aluminum square enclosed. Make a roll about 1 cm. in diameter—of the square, turning inward the blood without smearing it. Flatten one end of the roll and turn the edge over to prevent it from opening. *Allow the blood to dry*, then make a tight packet of the roll by flattening and turning over the other end. Fill out the data blank *in full*, return it with the foil packet and wire-loop to its envelope; place this in a larger envelope and mail to the Laboratory.

On receipt in the laboratory the flat packets are numbered with a pencil or blunt point. When they are opened the dried blood readily flakes from the foil. A portion is removed with a

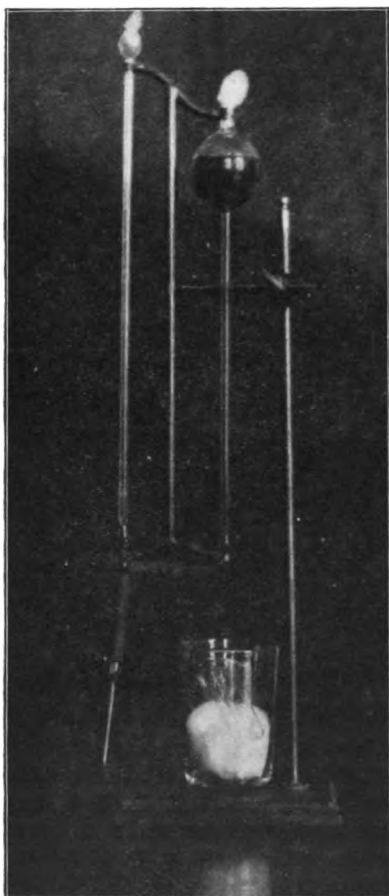


FIG. 6.

spatula, and 0.001 gramme is weighed on a fine balance. Two small squares of aluminum foil with rounded corners bent so as to form a shallow trough are kept on hand. They are of exactly the same weight, and used, one in each scale pan. One is used as a counterpoise. The small pieces of blood are dropped upon the other to be weighed and by means of a clean camel's hair brush, when weighed, are brushed into a small test tube. To the blood in the test tube, by means of a special pipette (Fig. 6), 0.1 c.c. of distilled water is added. This gives a dilution of 1:100 of the dried blood or 1:25 of the fluid blood. After shaking, the tube is allowed to stand for 30 minutes. From the supernatant fluid, by means of a large platinum loop kept for this purpose alone, a drop is placed upon a clean coverslip and by means of a very small platinum loop the drop is inoculated from a fresh broth

culture of *B. typhosus*. The loops are kept for this purpose alone and the very great difference in size renders the error in dilution practically negligible. At the end of two hours the presence or absence of the reaction is recorded.

The details of the collection, transmission and weighing of the

blood were elaborated by Dr. Louis B. Wilson and the method partially described in the *Philadelphia Medical Journal*, March 26, 1898.

The pipette shown in Fig. 6 is made by fusing a "T" tube into the end of an ordinary one c.c. water pipette graduated in hundredths. The bulb for containing the diluent is blown on an ordinary glass tube approximately of the same size as the pipette and bent as shown in the plate. The tube leading from the bulb is connected with the T piece of the pipette by a rubber tube and a glass bead is used in lieu of a pinch cock. This is also used between the end of the pipette and the dropper. The pipette may be filled to any desired point by means of the "bead-cock" and the desired amount allowed to escape from the pipette through the dropper into the test tube containing the dried blood. Care should be taken that there are no bubbles in the rubber tubes and that the dropper is absolutely filled with fluid. The metal frame, made in the laboratory workshop, holds the apparatus and renders it possible when the pipette and bulb are filled with fluid to sterilize the whole outfit in the autoclave. This combination of methods permits of accuracy and does not interfere with the operation of the laboratory over a wide territory since the drying of the blood permits of no changes in transit.

METHOD OF PREPARING RAPIDLY A SERUM FOR DIPHTHERIA DIAGNOSIS, WHICH WILL YIELD A MEDIUM OF CONSTANT QUALITY.

Large quantities of ox serum are collected at the slaughter house, brought to the laboratory and stored in tightly stoppered bottles with an excess of chloroform (approximately one per cent). These are kept cool and in the dark until a sufficient quantity of serum is accumulated.

The different lots of serum are blended in a large receptacle. To three parts of the serum is added one part of a one per cent glucose peptonized broth which contains five per cent glycerin.

This blended mixture, of sufficient quantity to provide for several months or a year, is bottled and tightly corked after chloroform in excess has been added to each bottle. It is then kept cool and in the dark until required for use.

When required the mixture is tubed and the tubes placed in a copper "sloper." Both front and back views of the "sloper" are shown in the accompanying photographs (Fig. 7). This "sloper" is made throughout of copper in order to facilitate rapid and even

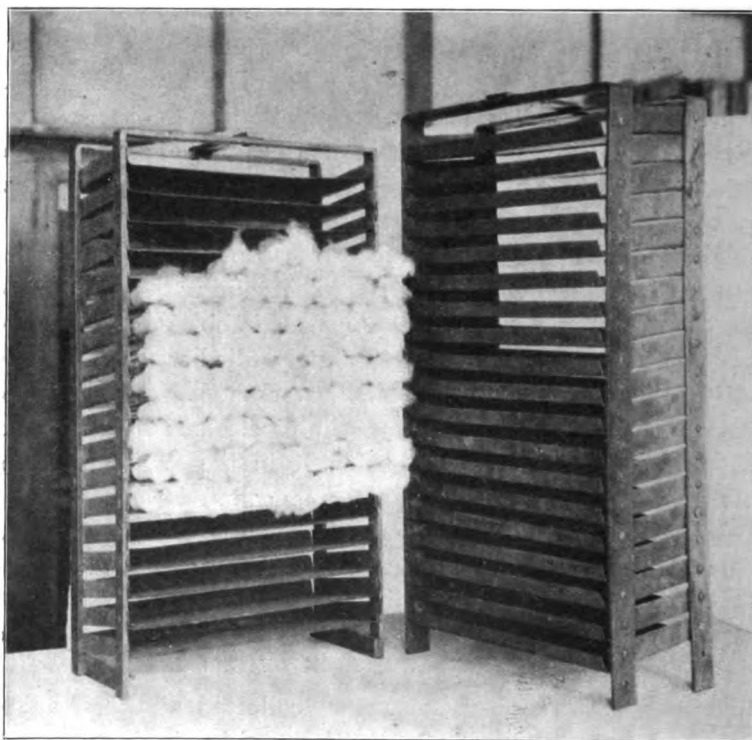


FIG. 7.

transmission of the heat. For convenience in filling, the "sloper" is tilted against the wall. It is so arranged that when it rests in its normal upright position, the requisite amount of slope is given to the serum in the tubes.

When all the tubes have been filled and placed in position, the "sloper" is set in an autoclave (Wiesnegg) which is *very gradually* heated with pin-point gas flame, the steam cock remaining open. If the flame is properly adjusted, at the end of

about one and one-half to two hours, steam begins to escape through the steam cock. The steam cock is closed 15 minutes after the steam has begun to escape and the flame under the autoclave is turned somewhat higher until at the end of a half hour a temperature of 120°C. is attained. This is maintained for 30 minutes when the flame is turned out. In the hands of the attendant, one heating yields serum ready for use which is smooth and free from bubbles. The blending of a year's stock gives a serum of practically constant composition for that period. The use of glycerin, which was begun early in our work with a view to preventing evaporation, has been continued, since all of our observations in relation to the diphtheria bacillus for the past seven years have been based upon cultures grown on this medium.

A MODIFIED DRIGALSKI-CONRADI CRYSTAL VIOLET MEDIUM.

This medium is a very stiff nutrient agar to which is added litmus solution and a solution of crystal violet. The method of preparation as modified by Dr. E. H. Beckman and used in the Minnesota State Board of Health Laboratory is as follows:

(a) *Nutrient agar*.—Add one liter of water to 680 grammes of finely-chopped lean beef and place in the cold for 24 hours. Express the juice and make up to one liter. Coagulate the albumin either by vigorous boiling for 10 minutes or by heating at 120°C. in the autoclave. Filter. Add 10 grammes of Witte's peptone, 10 grammes of nutrose, and 5 grammes of sodium chloride. Heat in the autoclave at a temperature of 120°C. for 30 minutes or boil vigorously for 15 minutes. Render slightly alkaline to litmus paper. Filter. Add 30 grammes of agar. Heat in the autoclave at a temperature of 120°C. for one-half hour or heat over the gas flame until the agar is dissolved. Render slightly alkaline to litmus paper while hot, if necessary. Filter through glass wool into a sterile vessel.

(b) To 130 c.c. of litmus solution (Kubel and Tiemann's) add 15 grammes of chemically pure lactose. Boil for 10 minutes.

(c) Mix (a) and (b) while hot. Render slightly alkaline to litmus paper, if necessary.

To the mixture add two c.c. of hot sterile solution of 10 per cent sodium hydrate in distilled water and 10 c.c. of a fresh solution of Höchst's crystal violet (0.1 gramme of crystal violet to 100 c.c. of sterile water).

The medium is now poured into Petri dishes and is of a deep purple color. So much water of condensation forms on the solidified surface that it is an advantage to use porous clay covers (Hill) for the Petri dishes instead of the ordinary glass cover. The medium keeps well but dries up rapidly.

After the medium has stood for 24 hours, it affords a smooth, firm surface upon which bacteria may be sown. The glass rod described in the original article¹ seems to be the best instrument for streaking out cultures.

The advantages of the medium are as follows:

1. The medium is not easily contaminated.
2. The acid colonies are easily distinguished.
3. Many of the common varieties of bacteria such as staphylococci and streptococci grow very slowly, if at all.
4. *B. typhosus* and *B. coli communis* grow rapidly and abundantly and the colonies of each can readily be distinguished. The larger size of the typhoid colonies, as stated in the original article,¹ has not been observed in this laboratory.
5. *B. typhosus* and *B. coli communis* can be obtained easily and rapidly in purity from mixed cultures.
6. All the colonies are on the surface and can be seen and transplanted with ease.

TESTS OF VALUE OF EMBALMING FLUIDS.

The Minnesota State Funeral Directors Association has had this matter under consideration for a number of years. The Association appointed a committee to investigate the efficiency of ordinary commercial embalming fluids with a view to determining and publishing suitable formulae for embalming purposes. An embalming fluid to be satisfactory should render inert all pathogenic bacteria in the cadaver without interference with the "cosmetic" effect which is so desirable in their work.

The Minnesota State Board of Health was requested to undertake the necessary laboratory investigation and the following

¹ *Ztschr. f. Hyg. u. Infektionskrankh.*, 1902, 39, p. 291.

methods are in the process of test and further elaboration by Dr. R. H. Mullin.

1. Test of germicidal efficiency. A mass culture of *B. typhosus* is made in sterile blood serum, pleuritic or other highly albuminous fluid comparable in nature to the body proteid content. Such culture after incubation for 24 hours is distributed in test tubes in 15 c.c. amounts. To each tube is added 0.8 c.c. of one of the embalming fluids. (The respective amounts above stated are employed because in embalming as ordinarily done, four quarts or eight pounds of embalming fluid is the maximum amount used for each 150 pound cadaver, in arterial injection.) After 24 hours, from each of the test tubes including controls, sub-cultures are made and results recorded. Those fluids which show germicidal properties according to the foregoing test are further investigated as follows:

2. Test of embalming qualities and "cosmetic" effects are made by the Embalmers' committee upon human cadavers.

3. Test of germicidal properties in embalming human cadavers may be used but more exact observations are possible by the use of rabbits which have succumbed to an inoculation of virulent anthrax bacilli so that every tissue and organ is filled with the living bacilli. The rabbits are weighed. Into each, through the left femoral artery, the embalming fluid under observation is injected in the proportion of 8 to 150 body weight. The injected animal is kept at ordinary temperature and at the end of 24 hours sowings are made from definite selected sites which are constant throughout the whole series of experiments. Those which are employed are brain, lung, liver, spleen, right gastrocnemius and contents of duodenum and of the ileum near the valve. Absolutely the same methods in operation and record are employed.

FIELD METHODS IN THE BACTERIOLOGY OF WATER.

In order to render it possible to begin the bacteriological examinations of water in the field so as to obviate changes during transmission to the laboratory, the following method has been evolved:

A supply of boxes is maintained for the transmission by express of the requisite materials to the site of investigation. Some of the details of the construction of the box and of its contents ready

for use together with the materials carried by the investigator into the field, may be seen from the accompanying photograph (Fig. 8). These boxes are made of pine, painted on the outside and loosely lined at top and bottom with corrugated paper. They are divided into three compartments by two upright partitions which slide into grooves. One end compartment contains Petri dishes which are wrapped in filter paper and sterilized. The paper in which they are sterilized protects them from breakage during transmission. The central compartment which is narrower than those at the ends, provides for two mushroom ground stoppered bottles of approximately 200 c.c. capacity. A small piece of twine is placed between the stoppers and the inside of the neck of the bottles. Clean muslin, in double or triple thickness, is placed over the stopper and tied in position with twine around the necks of the bottles; these are then sterilized in the autoclave. The twine between the stopper and the neck prevents the impaction of the stopper. In the other compartment, test tubes stoppered with cotton and containing plain agar and litmus lactose agar are carried. A case constructed of thin brass tubing with a slip cover is used to contain three water pipettes which are wrapped in thin paper in the brass case, the cover placed in position and the case and contents sterilized by heat. The hinges and hasps of the box are fastened in position with rivets and the box is so constructed that without mutilation, its contents cannot be disturbed by any one who does not possess a key to the padlock. All of the padlocks for these boxes and the larger boxes used for chemical samples, open by the same key.

The investigator carries with him a gasoline blast lamp (Turner's), a copper boiler which is carried in a leather slip case, a thermometer in a brass case (similar to that used for the pipettes), labels, glass pencils, etc.

Immediately after the collection of the samples, measured amounts are plated with plain and litmus lactose agar. The blast lamp is used for melting the tubes of agar in the copper boiler. After the plates are cool and firmly set they are re-wrapped in filter paper, inverted and packed in the box.

The colon bacillus determination is effected by adding one c.c.

of water to one c.c. double strength agar in the test tube. This agar thus diluted is allowed to set in the test tubes. The used test tubes are again replaced in their compartments, the box properly repacked, locked and shipped by express to the laboratory.

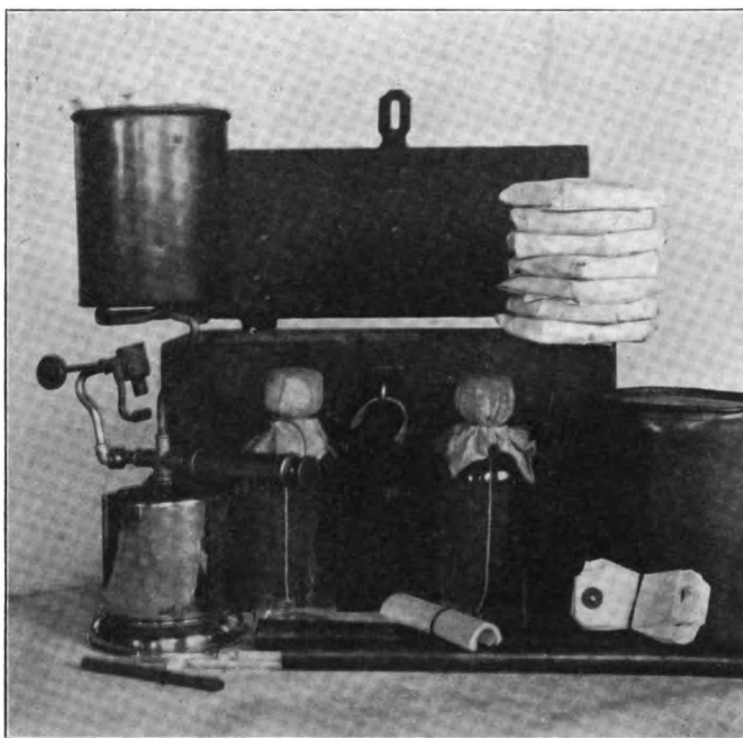


FIG. 8.

On arrival in the laboratory, the plain agar plates are placed at a temperature of 21°C . until ready for count and the litmus lactose agar plates are put immediately into the incubator at 37°C . The test tube containing the solidified agar sown with one c.c. of the water for the colon determination, is placed in the incubator after 10 c.c. of broth has been added and mixed with the agar which is thoroughly broken up with a stiff platinum inoculator. After 24 hours incubation, Smith tubes containing dextrose broth

and carbolized dextrose broth are inoculated from the test tubes containing the mixed agar and broth. From the Smith tubes colon bacillus may be isolated, if present, by streaking or plating. Other amounts of water may be similarly employed if desired. It is impossible by this method to use gelatin plates.

The particular advantages of the method are safety in shipment and protection against artificial contamination or loss of *B. coli communis* when present originally. The agar and litmus lactose agar plates having been inoculated with the water immediately after collection give a definite idea of the numbers and approximately, of the species of bacteria present in the water at the time of collection. These methods are employed in all the Minnesota work which is being carried on jointly by the United States Geological Survey and the State Board of Health and have been thoroughly tested by R. B. Dole, Assistant Engineer, U. S. Geological Survey and Dr. E. H. Beckman, Assistant Bacteriologist, Minnesota State Board of Health.

FILING METHODS FOR WATER DATA.

At the meeting of the Laboratory Section in Havana, sample files of the information collected in the joint work of the U. S. Geological Survey and the Minnesota State Board of Health were shown. It is impossible to reproduce the blanks and information at this time. The method consists in filing alphabetically according to locality, typewritten records of visits of inspection, which are supplemented by official statements on suitable blanks from health officers, city engineers, superintendents of waterworks, ice dealers, manufacturers, and others. Blue prints, drawings, photographs and other data illustrative of waterworks, sewerage, sewage disposal works, ice fields, possible sources of contamination, structural features and data concerning geological and meteorological features are filed together with the chemical, bacteriological and biological analyses. It is hoped in this way that the State Board of Health will ultimately have in its possession more complete information concerning each locality than will be possible, in most instances, for the locality itself to obtain and preserve.

AN IMPROVEMENT IN THE TECHNIC OF THE INDOL TEST.

JOSEPH MCFARLAND AND J. HAMILTON SMALL.

(The Medico-Chirurgical College of Philadelphia.)

SOME years ago Dr. Dunham introduced the peptone solution to facilitate the detection of indol, recommending it on the ground that its freedom from color made it more easily possible to detect the reddish tinge of the nitroso-indol than an amber colored fluid such as broth. It was, however, later shown by Theobald Smith that peptone solution is less well adapted to purposes of culture than broth, and after a careful study of the subject Smith recommended that the peptone solution be abandoned for the use of a sugar free broth.

It is customary to test for indol by the addition of a small quantity of a weak solution of potassium nitrite (0.01 per cent solution) and some chemically pure sulphuric acid, the liquid being shaken and the presence of a red color, in case very little is formed, noticed on the whitish froth. When the quantity of indol present is very small, a considerable delicacy of color perception is required to recognize it, so that any method becomes welcome that will concentrate the color at some particular portion of the tube. It has, therefore, occurred to us to endeavor to modify the test by the formation of a color ring, a slight modification of the usual method sufficing for this purpose.

The culture to be tested receives an addition of one drop of chemically pure sulphuric acid for each c.c. of fluid, this being well shaken. In case the microorganisms produce both indol and nitrites the red color now makes its appearance, as in the cholera spirilla, etc., but when the organisms produce no nitrites, as in the case of those of the colon group, after the sulphuric acid has been mixed with the fluid the dilute solution of potassium nitrite is allowed to trickle slowly down the side of the tube and form a layer on the surface of the fluid it already contains. The red color of the nitroso-indol now makes its appearance at the line of

contact of the two fluids where it is quite easy to recognize amounts of indol that could with difficulty be recognized should the tube be shaken and the color diffused through it.

By making solutions of indol crystals in distilled water and testing by this method, we have found it possible to recognize the presence of indol in dilutions of upwards of 1: 750,000. In dilutions of 1: 1,000,000 the color was not distinctly appreciable. In all lower dilutions the color was in proportion to the intensity of the solution.

Dr. Peckham made use of known dilutions of indol for determining the probable percentage of indol in different cultures, but the color being diffused throughout the entire liquid, the more delicate tints were lost.

It is quite easy to prepare a series of such color rings as have been described for making quantitative comparisons for ordinary laboratory work. We have found it quite satisfactory to make a series of test color rings in solutions containing two per cent of gelatin, the melted gelatin containing the indol and sulphuric acid is placed in a test tube and the melted gelatin containing the dilute nitrite solution is superimposed upon it. The color forms at the line of contact, as usual, and as the gelatin rapidly solidifies such tubes are not disturbed by handling or oversetting and can be kept for comparison for from 12 to 24 hours. Beyond 24 hours, the color begins to diffuse itself through the gelatin and is gradually lost.

**COPPER SULPHATE AS A GERMICIDE:
SOME NOTES ON ITS USE IN CONNECTION WITH SEWAGE
EFFLUENTS.**

GEORGE A. JOHNSON AND WILLIAM R. COPELAND,
Columbus, Ohio.

COPPER sulphate is a powerful germicide. Canning industries have taken advantage of this fact for a number of years, adding this chemical to meats and vegetables to prevent their fermentation by bacteria and other organisms. More recently copper sulphate has been used to destroy and prevent growths of algae in reservoirs; moreover, sanitarians are devoting considerable attention at the present time to the feasibility of using it to destroy bacterial life in polluted waters.

Following this general line tests are being carried on by the writers to determine the effect of copper sulphate when applied to effluents of sewage purification works. In this paper, certain of the results obtained to date, are presented. A more complete account of this work will be published in the near future.

METHOD OF CONDUCTING THE TESTS.

The experiments were conducted in 100 c.c. volumes, collected from the effluents of several different processes of sewage purification. The typhoid culture used was obtained from Parke, Davis and Company. The culture media employed were prepared in strict accordance with the recommendations of the Committee on Standard Methods of the American Public Health Association.

**GERMICIDAL EFFECT PRODUCED BY DIFFERENT CONCENTRATIONS
OF COPPER SULPHATE.**

In testing the germicidal power of different concentrations of copper sulphate, a solution of the commercial article was added in the proportion of one part of the chemical to 200,000, 100,000, 50,000 and 25,000 parts, respectively, of sewage effluents. To some of the samples large numbers of typhoid bacilli were added, before introducing the chemical.

The results presented in Table 1 show that the bacteriocidal power of the copper sulphate increased with the concentration of the solution. It is to be noted, however, that complete sterilization was not effected in 24 hours in any of the samples.

TABLE 1.
GERMICIDAL EFFECT OF VARIOUS CONCENTRATIONS OF COPPER SULPHATE.

PARTS OF COPPER SULPHATE TO PARTS OF SEWAGE EFFLUENT	BACTERIA PER C.C.		
	Before Adding Copper Sulphate	3 Hours After Adding Copper Sulphate	24 Hours After Adding Copper Sulphate
1 to 200,000	1,400,000*	120,000	1,100
1 to 100,000	1,400,000*	28,000	650
1 to 50,000	1,400,000*	20,000	600
1 to 100,000	240,000§	150,000	800
1 to 50,000	240,000§	84,000	250
1 to 25,000	210,000§	33,000	220

* Average of four sets of results.

§ Average of three sets of results.

RELATION BETWEEN THE PERIOD OF CONTACT AND THE GERMICIDAL ACTION OF COPPER SULPHATE.

As will be seen from the figures given in Table 2, the numbers of bacteria diminished very rapidly during the first hour of contact. The stronger concentrations destroyed the organisms somewhat more rapidly than the weaker.

TABLE 2.
RAPIDITY OF THE GERMICIDAL ACTION OF COPPER SULPHATE.

BEFORE ADDING COPPER SULPHATE	BACTERIA PER C.C.				
	15 Minutes	30 Minutes	1 Hour	3 Hours	15 Hours
910,000*	460,000	270,000	240,000	80,000	700
880,000§	250,000	220,000	190,000	47,000	500

* In this set the proportion of copper sulphate to effluent was 1 to 100,000.

§ In this set the proportion of copper sulphate to effluent was 1 to 50,000.

Typhoid bacilli were added to these samples before they were submitted to the test, the number of this species largely predominating. In each case the results given are averages of seven sets of experiments.

EFFECT OF TEMPERATURE ON THE RAPIDITY OF THE GERMICIDAL ACTION OF COPPER SULPHATE.

As is well known, chemical reagents exercise their characteristic properties to a much greater degree at some temperatures than at others. Copper sulphate, for instance, is more active as a germicide at temperatures of about 20° C. than at materially lower temperatures. The effluents of sewage disposal works will vary widely in temperature throughout the year. In order to obtain information on this point, one-half of a series of samples was kept at a temperature of 20° C., and the other half of the series at about 5° C. Copper sulphate was added in different concentrations, and tests for the numbers of bacteria were made at intervals.

The results of these tests are given in Table 3, and show clearly that the bacteria disappeared more rapidly at the higher temperature. Further, it is worthy of note, that at the higher temperatures, particularly in the case of the lower concentrations, the power of the germicide appears to have become exhausted soon after the sixth hour of contact.

TABLE 3.

EFFECT OF TEMPERATURE ON THE GERMICIDAL POWER OF COPPER SULPHATE.

TEMPERATURE DEGREES C.	PARTS OF COPPER SULPHATE TO PARTS OF SEWAGE EFFLUENT	BEFORE ADDING COPPER SULPHATE	BACTERIA PER C.C. AFTER STANDING IN CONTACT WITH COPPER SULPHATE FOR			
			30 Minutes	2 Hours	6 Hours	18 Hours
5	1 to 100,000	460,000	100,000	51,000	24,000	2,600
20	1 to 100,000	600,000	44,000	3,300	700	81,000
5	1 to 50,000	520,000	48,000	34,000	3,900	1,800
20	1 to 50,000	580,000	18,000	2,900	650	3,900
5	1 to 25,000	490,000	37,000	24,000	5,500	1,300
20	1 to 25,000	580,000	15,000	1,300	250	350

No typhoid bacilli were added to these samples. The figures given are in each case the average of three sets of experiments.

EFFECT OF ORGANIC MATTER, AND OF DISSOLVED CARBONATES OF LIME AND MAGNESIA, ON THE GERMICIDAL POWER OF COPPER SULPHATE.

Effluents of sewage purification works contain more or less organic matter, which probably affects the germicidal power of

copper sulphate to some extent, through absorption. The carbonates of lime and magnesia, dissolved in some sewage effluents, probably affect a considerable precipitation of the copper as a basic carbonate, in which state it is less efficient as a germicide. While the results presented in Table 4 do not show the individual effect of these factors, it is nevertheless evident that the germicidal power of the copper sulphate was less active in the samples of sewage effluent containing organic matter and dissolved carbonates, than in redistilled water which was free from both.

TABLE 4.
EFFECT OF ORGANIC MATTER AND DISSOLVED CARBONATES ON THE GERMICIDAL POWER OF COPPER SULPHATE.

CHARACTER OF SAMPLE	TEMPERATURE DEGREES C.	PARTS PER MILLION		BACTERIA PER C.C.				
		Organic Nitrogen	Dissolved Carbonates	Before Adding Copper Sulphate	After Standing in Contact with Copper Sulphate for			
					5 Min.	1 Hour	3 Hrs.	15 Hrs.
Sewage effluent.	20	2.87	233	1,300,000	830,000	260,000	21,000	600
Distilled water..	20	0	0	1,300,000	420,000	19,000	650	11

Large numbers of typhoid bacilli were added to these samples. The copper sulphate was used in the proportion of 1 part to 50,000 parts of the samples.

OTHER DISTURBING FACTORS.

In the consideration of the efficiency of copper sulphate as a germicide in connection with sewage effluents there are other disturbing factors which must be borne in mind, as: for instance, sulphides which, by precipitating the copper, would probably diminish its germicidal power.

REGARDING COSTS.

The figures given in the preceding tables indicate that there are a number of important factors which must be taken into consideration in connection with the use of copper sulphate as a germicide. They affect its efficiency, and point to the fact that the numbers of bacteria are likely to diminish so slowly where only one part of copper sulphate is used in 100,000, that the germicide must be added in greater quantities to effect a sterilization of the sewage effluents within a reasonable period of time.

If it shall be subsequently proved that it is necessary to use materially higher concentrations of this chemical than 1 to 100,000, the question of the cost of the treatment will be brought into special prominence.

At the present time the cost of copper sulphate is in the neighborhood of \$120 per ton. Assuming that this figure is correct, the cost for chemical alone is shown in the following table:

COST OF COPPER SULPHATE.

Concentration	Grains per Gallon	Pounds per Million Gallons	Cost for Chemical per Million Gallons at \$0.06 per Pound
1 to 100,000585	83.6	\$ 5.02
1 to 50,000	1.17	167.2	10.04
1 to 25,000	2.34	334.4	20.08

METAMORPHOSIS OF FILARIA IN THE BODY OF THE MOSQUITO (*CULEX PIPIENS*).

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INTRODUCTION.

THE names of Demarquay, Lewis, Manson, Cobbold, Low and James may be said to epitomize the history of filaria. They may be divided into two groups; one representing the fortunate discoverers of these curious nematodes, and the other, including Manson, Low and James, the students of the transformations of the parasite in the mosquito.

If we consider that filaria, as demonstrated by Manson, is transmitted by the mosquito, we are disposed to conclude that the prevention of its propagation must be an easy matter; and, theoretically, it is so, provided we can secure a strict isolation of infected patients from the bite of mosquitoes.

There are still some points that require further elucidation, such as the mode of transmission from the mosquito to man, but, from the sanitary viewpoint, it is sufficient to know that the evolution of the filaria embryo occurs exclusively in the mosquito. This knowledge has not been applied in sanitary practice.

My own investigations have concerned themselves with the evolution of the parasite in the body of the mosquito, and with the determination of how, where, and when the worm leaves the body of the insect. My aim has been to include the whole process, and to leave no doubt as to the inoculation of filaria by mosquitoes in the human subject.

SOURCE OF MATERIAL.

The case utilized for my experiments was one of moderate infection. He was sent to me by Dr. Enrique Nuñez and he subjected himself patiently to many annoying procedures.

The clinical history is briefly as follows:

He had always enjoyed good health, except that he was operated on in June 1903, by Dr. Nuñez, for a left inguinal hernia.

He remembers to have had, since he was eight years old, a slight enlargement of the glands in the right groin. This increased very gradually, extending downwards into the thigh.

Recently the diagnosis of adenolymphocoele was made, and the tumor was totally extirpated in January, 1904.

In July, an abscess, about the size of a hen's egg, formed in the left thigh. This was opened, and healed in about nine days. An examination of the blood made at this time revealed a considerable number of filaria embryos.

On the day of admission in "Las Animas" hospital, August 22, 1904, the blood was examined at 8:30 p. m. with negative results. On the following day, at 9:30 p. m. from two to three embryos were found in each preparation. A little later we could find from 10 to 12 in each preparation. We may look upon this as the average during the nights when mosquitoes have been applied.

MOSQUITOES, AND TECHNIQUE OF THEIR INFECTION WITH FILARIAE.

Mosquitoes were applied to our patient on seven occasions, separated by the intervals of time necessary for the study of the infected insects. The conclusions of the present paper are founded on the first six applications. The seventh series is still under observation.

The facilities for this line of experiments at "Las Animas" Hospital are great, since we have rooms well screened with wire gauze, and assistants who have been well trained in mosquito work.*

Several kinds of mosquitoes have been used in these experiments, but the one in which we have been able to follow methodically the phases of development of filaria has been *Culex pipiens*. This particular species must be allowed to bite in condition of relative freedom, under a mosquito net. Our *Anopheles (albipes)*, *Stegomyia*, and *Culex consobrinus* on the other hand, will bite readily in jars. The insects were made to bite at night, in accordance with the law of periodicity that causes the *filaria nocturna* to appear in the peripheral circulation only at night.

In order to obtain infection of the insects by the greatest number of embryos possible, it is advisable to have the patient go to bed, at least two hours before the mosquitoes are let loose under the net. Our failure to comply with this requirement caused a marked diminution of the infection in one of our series.

After remaining under the net all night with the patient, the insects were collected on the following morning.

*I have to thank Mr. E. Gros, the assistant in the laboratory, for valuable aid in carrying out these investigations.

The hour of 12 P. M. was fixed upon as the time of beginning of the infection. By that time all the insects had surely bitten.

The mosquitoes employed were mostly specimens reared in captivity. Some insects were captured in the attendants' dormitories, where there was no filariasis. There can be no doubt, therefore, that the mosquitoes had not been previously infected.

A curious difference is noticeable between the insects proceeding from these two sources. The insects reared in our breeding jars are much less likely to bite than those captured in adult life. In the former class I have had only two out of 50 insects bite on some occasions; whereas hungry veterans captured in adult life are always successful biters.

Of 11 insects of the species *Culex consobrinus*, that had bitten the patient, not one became infected.

Out of 19 *stegomyias*, only one showed some infection, in the shape of two filariae that were still alive on the fifth day, but poorly developed.

Out of 70 specimens of *Culex pipiens* only eight failed to become infected, and these had been applied under the unfavorable conditions previously mentioned.

Of the varieties experimented with, *Culex pipiens* is without doubt the transmitter of this infection.

As bases for the conclusions arrived at in this paper, I wish to mention the following facts:

1. The female alone is capable of sucking blood.
2. The object of the blood is to assist in ovulation.
3. The insect does not perish after laying her eggs.
4. The duration of life of *Culex pipiens* is generally about two or three months.
5. The insect may bite every four or five days. It digests its blood meal in three or four days.
6. The female mosquito, kept in captivity, feeds after its first blood meal, on several kinds of food. Some experimenters feed them on fruit. In our laboratory we use sugar. A female mosquito will feed on sugar before she has digested the blood. I have dissected insects whose true stomach still contained blood and whose accessory stomach, or esophageal diverticulum, was full of syrup.

HISTOLOGICAL TECHNIQUE.

For studying the distribution of the filariae in the body of the mosquito, sections are best used.

On the other hand, if we wish to follow the cycle of development of the parasite, of its dimensions and structure, we must dissect the infected mosquitoes.

TECHNIQUE FOR THE PREPARATION OF SECTIONS.

1. The live mosquito is placed in absolute alcohol. In this manner the penetration necessary for fixation and dehydration is obtained. Duration 24 hours.
2. Removal of wings and legs in the same liquid, in a watch glass.
3. Equal parts of absolute alcohol and ether, 24 hours.
4. Weak solution of celloidin, 24 hours.
5. Thick celloidin, 24 hours.
6. Mounting on block.
7. Chloroform, 20 minutes.
8. Eighty per cent alcohol until ready to cut.

The sections were always stained in Böhmers hematoxylin, followed in some cases by eosin and in others by Van Gieson's solution.

DISSECTION.

The living insect is placed in a test-tube with a small amount of water, and is shaken until the wings become wet, and the insect floats on the water. The liquid is poured into a watch glass, where the legs and wings are pulled off by means of forceps.

The further dissection should be made in the following order:

Dissection of the abdomen upon a slide, constantly irrigating the mosquito with distilled water or weak salt solution. After dissection place under microscope and determine by medium power whether there are any filariae. The head and thorax are transferred to another slide. Here the chitin is broken near the neck, and the head is transferred to another slide. Each part is thus dissected separately, and we are able to determine the localization of the parasites. If the latter be well advanced in their development, they push their way out through the openings made in the chitinous covering at the time of dissection.

If we wish to preserve the specimens, the filariae, if they are large enough, are transferred from place to place upon the slide, and repeatedly washed, while we clean and remove all detritus of the body of the mosquito from the slide. We finally add a drop of mixture of water, glycerin, and formalin, and the preparation is covered and cemented. Such preparations may be studied with the immersion lens.

When the filariae are too small to be isolated, it is best to leave them in the midst of the detritus, and to stain them as follows:

1. Allow them to dry on the slide.
2. Fix with 95 per cent alcohol, 1 to 2 minutes.
3. Place in water a few minutes.
4. Böhmers hematoxylin, 1, 2, or 3 minutes, according to the intensity of the stain desired.
5. Wash in running water, 3 to 5 minutes.
6. Watery .01 per cent solution of eosin, $\frac{1}{2}$ to 1 minute.

7. Wash in 85 per cent alcohol.
8. Dry with paper.
9. Oil of cloves, 2 to 3 minutes.
10. Xylol.
11. Balsam.

The above technique may appear complicated, but it may be carried through in 8 to 10 minutes. Without staining, the filariae, when they are too small or too few, may escape observation.

The dissection above described is not a careful dissection of the insect, but should be considered rather as a coarse fragmentation into sections, as follows: the stomach, intestine and ovaries in one piece; the thoracic muscle in four or more bundles; and the head and mouth parts. Care should be exercised not to lose a single fragment.

METAMORPHOSIS OF FILARIA IN MOSQUITO.

I shall describe now the several steps in the metamorphosis of the filaria in the mosquito. I shall touch upon some points that have been in dispute, but upon which my opinions are free from all bias, since my observations were made before I had a complete knowledge of the literature.

CHANGES OF POSITION OF THE FILARIA WITHIN ITS HOST, AND THE DATES COUNTING FROM THE FIRST DAY OF INFECTION, THAT CORRESPOND TO SUCH CHANGES.

Histologically we may say that the filariae are found in one or the other of two structures, namely: in the stomach, together with the blood ingested from an infected patient; or in the loose connective tissue, called the fat body.

Topographically we find that filaria embryos may occupy the following positions: (1) they are taken into the stomach where they remain during a variable number of hours; (2) they then pass to the thorax where they lie as in a nest, undergoing the principal morphological changes; and (3) they usually pass out through the head and labium, but sometimes the worm loses its way in the body and becomes lodged in the abdomen under the chitinous covering.

The transformations of the filaria may be considered as follows in my several series:

First and second series.—At the end of 36 hours all the embryos had left the cavity of the stomach, and had wandered into the thorax. At the end of 12 days they were found in the neck.

Third and fourth series.—In 32 to 40 hours the embryos had passed to the thorax. As an example of unusual precocity I may mention one instance in which an embryo had found its way to the thorax 13 hours after the ingestion of blood in the stomach.

In these series filariae reached the neck 13 days and 15 hours, and the labium 15 days and 14 hours after infection (stained preparations similar to those of Low). On the 20th, 30th and 38th day filariae were still found in

the labium. One of the insects of this series presented, 15 days and nine hours after infection, 10 flariae in the head, and only three were still in the thorax.

Fifth series.—As in the other series nearly all the embryos had already left the stomach at the end of the first 40 hours. I should mention, however, one mosquito in whose thorax 21 flariae were found, and whose stomach filled with liquid blood still contained eight worms three days and nine hours after infection.

In this series the worms were still found in the thorax after 15 days and 14 hours, and their metamorphosis was not quite completed. They reached the head at the end of 19 days and nine hours. On the 30th and 38th day they were still visible under the microscope, moving in the labium of the living insect.

Sixth series.—In this series the flariae reached the head after a period of 22 days and eight hours.

To recapitulate: In the first four series the flariae reached the head in 15 to 16 days. In the fifth series some delay was noticed, since flariae were found in the stomach after three days and nine hours, and none were found in the head on the 17th day. In the sixth series the worms reached the head and labium on the 22d day.

This difference in the time of migration is due to the fact that the embryos do not migrate until they have completed certain phases of their cycle of development. We shall consider later on the cause of this delay of development in some cases.

DIMENSIONS AND GENERAL CONFIGURATION OF THE EMBRYOS IN THE DIFFERENT STAGES OF DEVELOPMENT.

The morphological changes were found to vary in the different series in the same manner as the migrations of the worm.

In the following table all the series are presented together, and the maximum time, that corresponds with the several changes, is given.

The dimensions of the embryo filaria in the human blood are: length, from 0.130 to 0.300 mm; width, 0.007 to 0.011mm.¹

DIMENSIONS OF THE FILARIA EMBRYOS.

	Length	Width
In the stomach of the mosquitoes a few hours after ingestion - - - -	0.203-0.277 mm.	0.0055-0.0074 mm.
In the body of the mosquitoes during the first three days - - - - }	{ 0.240 mm. - -	0.0121 mm.
	{ 0.208 mm. - -	0.0111 mm.
	{ 0.203 mm. - -	0.0148 mm.
In the three following days up to the sixth day - - - - }	{ 0.192 mm. - -	0.0185 mm.
	{ 0.176 mm. - -	0.0194 mm.
	{ 0.160 mm. - -	0.0185 mm.
In the three following days up to tenth day - - - - }	{ 0.284 mm. - -	0.037 mm.
	{ 0.280 mm. - -	0.0407 mm.
	{ 0.336 mm. - -	0.0333 mm.
During the following days the filaria continued to grow until it reached its full development on the 12th day in the more rapid, and the 22d in the slower series - - - - }	{ 1.408 mm. - -	0.024 mm.
	{ 1.440 mm. - -	0.024 mm.
	{ 1.504 mm. - -	0.032 mm.

¹ Dr. M. Braun, *Die Thierischen Parasiten d. Menschen*, Würzburg, 1903, p. 265.

A careful study of the above figures will show that the development of the worm occurs in two stages. During the first stage the embryo grows shorter until it measures 0.160 mm., and then it grows longer, and attains the maximum of 1.504 mm. The width, on the other hand, changes in the opposite direction: during the first stage, it increases from 0.012 mm. to 0.018 mm. Finally, both dimensions increase; the length, much more rapidly than the width.

With these changes in size we have also morphological changes. The shape of the embryo in the human blood is slender and delicate. After the ecdysis in the stomach of the mosquito, and soon after the migration to the thorax, the embryos become plump and sausage-like. Towards the end of the cycle of development the worm grows gradually longer and, though much larger, assumes again its original shape.

STRUCTURAL CHANGES DURING THE CYCLE OF DEVELOPMENT.

Fig. 1, A, gives a good idea of the hyaline sheath, fitting closely around the body of the embryo, but extending considerably beyond its length.

The embryo presents a somewhat square rather than rounded head. The posterior extremity is quite pointed. About one-third or one-fourth the length of the worm from the anterior extremity there is a clear space, the V-shaped space described by Manson. There is much doubt as to the embryonic significance of this space. A similar one is also found towards the posterior extremity. These spaces are quite apparent in stained preparations. In fresh preparations they appear as highly refractive spots.

In living embryos there is a remarkable series of refractive points or granules extending, rosary-like, from the middle of the embryo to about one-quarter from the posterior extremity. The whole rosary moves backwards and forwards, but never advances beyond the center of the embryo.

The examination with high powers reveals a distinct transverse striation of the body (Fig. 2).

The anterior extremity or mouth has been described in divers manners. The highly refractive character of this region gives rise to much uncertainty. I have not been able to form any positive opinions as to the character of the structure. In one instance only was I convinced of the existence of an arrangement consisting of three prismatic teeth. These teeth had the appearance and color of the teeth found in the maxillae of mosquitoes and somewhat yellowish color, such as is found in chitinous structures. The observation, however, is too isolated to warrant any final conclusions. The mobility of the living worm renders the observation more difficult. In stained preparations we see simply a sheath filled with minute granules.

The posterior extremity becomes remarkably changed during the metamorphosis. From being sharp and pointed, it becomes the widest part of the worm.

Upon leaving the stomach of the mosquito, the filaria leaves its sheath in the coagulated blood. This operation is called ecdysis.

The first change visible after arrival in the thoracic muscles is a slight narrowing of the body of the embryo near its posterior fifth or sixth. This progresses slowly, and, at the same time, there is a gradual invagination of the

posterior sixth, into the anterior portion. The worm grows shorter and thicker (Fig. 3).

The invagination continues until there is only a small portion of the pointed extremity protruding, like a small appendix from the thickened and rounded extremity.

The anterior extremity is somewhat square-shaped. The mouth, at this time, constitutes a round opening leading into a funnel-shaped cavity. At the narrow termination of the latter we find the beginning of the digestive tract. This is now perfectly visible, extending to the anus. It becomes, however, more prominent when the filaria begins to elongate.

During this stage of elongation, the cephalic extremity does not change; excepting that, in some instances, a small, stiletto-like body is seen projecting from the oral funnel. The posterior or appendicular extremity suffers a retrograde metamorphosis. Although the worm has lost its outer sheath in the process of ecdysis, it is still surrounded by an adhering hyaline membrane. This fine integument, first described by James, becomes quite visible when it swells by imbibition. The structures contained within this membrane, at the end of the appendix, are seen to disappear, leaving an empty portion at the point. This portion appears then in the shape of a very small triangular, hyaline cap, upon the rounded posterior extremity.

The intestine can now be seen consisting of three distinct portions: one extending 0.384 mm. backward from the bottom of the oral funnel. Here we find a constriction which reminds us of the esophageal bulb. From this point the intestine continues, forming a slightly undulating line to the anus. The latter appears at varying distances from the tail end, as may be seen in the following table:

Length of Filaria	Width of Same	Distance from Anus to Tail End
0.192 mm.	0.0185 mm.	0.021-0.0240 mm.
0.336 mm.	0.0333 mm.	0.0259 mm.
0.640 mm.	0.0370 mm.	0.0444 mm.
1.344 mm.	0.0320 mm.	0.056-0.0690 mm.

Apparently the distance of the anus from the cauda increases; but in relation to the increase in length of the worm the distance really diminishes.

The diameter of the anal opening is greatest when the filaria is shortest; it becomes narrower and filiform as the embryo reaches its complete development in the mosquito.

I have seen a peculiar viscid substance leaving the anus, and remaining adherent to the filaria for some time. The substance appears at times to be inclosed in transparent vesicles.

As the embryo reaches its maximum length, the final changes occur in the caudal end; namely, the development of the three lobes. These are at first very small, but become later quite prominent. Sometimes they give the impression of a hook formation; but they are rounded, and symmetrically arranged around the point.

I desire to call attention to a peculiar structure observed by me in three of the embryos examined. It was observed in the stage just preceding the final development. I do not mention it as a regular stage in the growth of the worm because I have only observed it in a few instances.

This peculiar structure presents itself at the caudal extremity. It appears in the shape of a tube which starting from the posterior end, extends forwards, bending somewhat, and protruding sidewise at a short distance from the anus, where it projects forming a small conical protuberance (Fig. 4). The whole tube can be seen through the transparent tissues, and presents the shape of a sleeve, the cuff of which forms the conical elevation. This peculiar structure is quite motile. By movements that may be described as erectile the sleeve-like organ is seen to bend, causing a lateral swelling of the terminal portion of the body of the filaria. At the same time the small titlike protuberance moves nearer or further from the anus. In this protuberance we are able to observe that the organ in question is hollow.

In a filaria measuring 1.344 mm. in length, and 0.032 mm. in width the following measurements were made (Fig. 4):

Distance from the anus (a) to the caudal extremity (b).....	0.069 mm.
Distance from the anus (a) to the nearest edge of the protruding cone (c).....	0.009 mm.
Distance from the tail end (b) to the nearest edge of the protruding cone (c).....	0.045 mm.
Width of the protruding cone at its base.....	0.015 mm.

The titlike protuberance measures 0.0105 mm. in height on the caudal side, and 0.006 mm. on the side toward the anus.

During the erection the distension of the posterior portion always occurs on the side opposite the titlike projection. This expansion may extend to a distance from the tail end varying from 0.0525 mm. to 0.069 mm.

In measuring from the tail end the rounded extremity, not the appendix, was taken.

These measurements were made on living filariae anesthetized by adding a few drops of ether to the water.

I am not able to give any functional significance to this structure. I am sure that it is independent of the anus. Though no spicules are found in connection with the organ, the idea suggests itself that it may have some relation to the sexual organs.

The description of the steps in the metamorphosis of the embryo in the *Culex pipiens* may be condensed as follows:

1. The insect sucks the blood of a patient infected with filaria.
2. The embryo loses its sheath in the stomach of the mosquito, ecdysis.
3. Migration from the stomach to the thorax. This migration always takes place through the gastric wall, since both orifices of the gastric dilatation are completely closed when the stomach is full. The embryo leaves its sheath in the gastric contents or caught in the wall of the stomach, where it is left at the moment of exit.

4. The embryo rests in the thorax, and goes through the following transformations:

- (a) Narrowing and invagination of the tail.
- (b) Invagination continues and the embryo grows shorter and wider.
- (c) Widening and shortening continue, and the invaginated portion forms a hyaline appendix.
- (d) Period of growth and formation of the three lobes.

The motility and non-motility of the embryo constitute biological features that are characteristic for the various phases of its development.

The active motility of the worm in the human circulation is further increased when the worm reaches the stomach of the mosquito. Upon reaching the thorax, however, all active motion ceases, and we only observe now and then very slight and sudden lateral movements. When the worm is nearing the maximum of its growth we notice a slight increase in the lateral movements of the anterior extremity. As soon, however, as the embryo has completed its metamorphosis in the body of the mosquito, it recovers its motility, in order to accomplish its migration to the head. There the filaria awaits the opportunity to complete its cycle of development in the human host.

This latter motility is peculiar in that it enables the worm to push its way in the midst of soft tissues. It is a kind of motion that is quite distinct from that of the earlier stages when the animal has to move in liquids. I shall dwell further on upon these differences and shall use them in support of my views.

The worm, then, shows its activity in order to enter and to leave the thorax, and while in the latter it lies quiescent during its metamorphosis.

It happens sometimes, though rarely, that when the filaria reaches its maximum size, and starts on its way to the head, it may mistake the route, and wander towards the caudal extremity. The worm, however, will always keep in the fatty tissue, and close to the chitinous covering. These stray worms all proceed from the thorax. I have never met with a single embryo undergoing the process of metamorphosis in any other structure than the thoracic muscles.

From three principal characters we may conclude that the filaria has completed its cycle of development in the mosquito, namely:

1. The arrival in the labium.
2. Complete development of the three lobes in the caudal extremity.
3. Active motility.

I desire now to consider the marked differences that were noticed, with respect to the time consumed in the cycle of development in my several series.

In his earlier investigations Manson saw embryos already in active motion seven days after infection. Bancroft¹ says he never saw them before the 16th or 17th day, and in cold weather, even the 20th; James² asserts that from 12 to 14 days are required for the complete metamorphosis, but he admits that, in localities where filaria abounds there may be species of mosquitoes that are peculiarly favorable to the development of the embryo. In this way he explains the seven days mentioned by Manson in his earlier work. He mentions the influence that climate and other factors may have upon these changes, and he reminds us of the fact that some of Manson's mosquitoes were kept in the incubator at temperatures ranging between 27° C. and 29.5° C.

We should not forget that the different results above mentioned were obtained in different countries. On the other hand I have also obtained the same variability in the results, though I was working in one locality, in the same laboratory, and with the same kind of mosquitoes.

The different results obtained by me, experimenting always with the same kind of mosquitoes, show that we cannot accept the suggestion of James to the effect that there may be species of mosquitoes that favor the development of the filaria.

Different species of mosquitoes may serve or not as hosts, but if the insect is capable of acting as such, the time limit of the cycle extends within bounds that have not yet been fixed.

In my series of experiments the only variable point was the season of the year. The results obtained were the same for each

¹THOS. L. BANCROFT, *Jour. of Trop. Med.*, 1899, 2, p. 96.

²S. P. JAMES, *Jour. of Trop. Med.*, 1900, 3, p. 46.

series and the variations were noticeable only in the comparison of one series with another. The variations correspond, therefore, with the changes of temperature that prevailed at the time of experimenting with each series.

From the official bulletin of the meteorological station I have been able to obtain the mean temperature prevailing during the time of each series of observations, and I present the results in the following table.

MOSQUITOES (*Culex pipiens*).

Series	Dates	Complete development of the filaria in	Mean temperature for each period
1, 2, 3.....	August 25 to Sept. 28 {	15 days	25.5° C.
4.....	October 12 to Oct. 27 {		
5.....	Nov. 9 to Nov. 26 {	19 to 23 days	22.5° C. } — 21.8° C.
6.....	December 13 to Jan. 4 }		21.2° C. }

It is evident from the above table that the temperature is the chief factor in modifying the cycle of development. Heat is, then, the climatic condition that favors the development of the embryo.

The daily oscillations of the temperature were nearly the same during the time of the several series. The daily range was of 4.4°, 4.5° and 4.1°. The daily oscillations in Cuba, therefore, do not influence the growth of the worm.

I am preparing a series of experiments with mosquitoes kept in incubators at a uniform temperature, in order to fix the extremes of most favorable, and absolutely unfavorable temperatures.

THE ESCAPE OF THE WORM FROM THE INTERMEDIARY HOSTS.

The several kinds of living beings that may act as transmitters of disease, may be divided into two great groups. In one the infecting agent must go through a cycle of development within the host, without which the further transmission to another animal is impossible. In the other group the parasite, without undergoing any developmental changes is mechanically carried from one animal to another by an intermediary animal acting as an indifferent vehicle.

However, the act of infection by the first group of hosts may occur either actively or passively. In the first instance the con-

veying animal, by its own act, inoculates the infecting agent; in the second, the intermediary host remains passive until the animal to be infected, takes up the infecting agent. An illustration of the first, or active mode of transmission, we have in the mosquito inoculating filaria by its own bite. We have in the hog, in whose flesh the cysticercus must wait passively until it is swallowed by man, an illustration of the second or passive mode of conveyance.

The filaria, leaving the body of man in the embryonal stage of its development must suffer a series of changes that can only take place in the body of the mosquito, before the worm can reach maturity, adult and sexual life, once more in the body of man; the mosquito (in my experiments *Culex pipiens*), is therefore the only transmitting agent.

When we take up the problem as to how this transmission is carried out, we find differences of opinion: some holding that the process is a passive one, and others that it is active. In the first instance it is maintained that the mature embryo passes from the body of the mosquito into drinking water, and is finally swallowed by man; in the second instance it is maintained that the mature embryo is directly inoculated by the mosquito. The former is the the old theory of Manson; the latter, the more modern of Low and James.

In attempting the solution of these problems, we must start with a thorough understanding of the facts presented in the earlier parts of this paper, specially with respect to the evidences of the final development of the embryo, its arrival in the labium, and the time that has passed since the mosquito used in the investigation became infected.

With all this information, I proceeded first to determine whether the filaria leaves spontaneously the body of the mosquito when the latter, still living, drops into the water, and care is taken that the integrity of the structure of the insect is preserved.

With this object in view I selected mosquitoes whose date of infection was well known, and in which the stage of development of the filariae was well established by the examination of other insects of the same series.

The selected mosquitoes were placed in water in a watch-glass, and studied under the microscope. The results were always negative. The mosquitoes died without discharging a single worm. Upon dissection of these insects at the end of 24 or 28 hours, all the filariae within them were found dead, which is also the case when they are allowed to remain a few hours (up to 18) free in water.

That the integument of the insect was preserved in these cases was shown by the exit of large numbers of infusoria as soon as the chitinous covering was broken in the act of dissection.

This experience proves that, when a mosquito falls into the water if its cuticle be preserved, the filariae it may contain are unable to escape and perish by imbibition of water within a period of 24 hours.

My second series of experiments was intended to determine whether the filaria escapes at the time when the insect is feeding, or drinking, or when it lays its eggs. Be it understood that in these acts we exclude especially the sucking of blood.

Six mosquitoes, from among those most seriously infected, were placed in a separate jar on the sixth day of their infection. The water in the jar was examined every day.* The lumps of sugar hung up in little bags to feed the mosquitoes were also examined daily by dissolving them. The results of these experiments were always negative even up to the 38th day. At the end of this time while no filariae were found in the water, they were still to be found, living, and active, in the heads of the mosquitoes.

In other mosquitoes of the same series dissected at stated intervals, we were able to see that the filariae were fully developed, and had arrived in the labium on the 17th day.

My second experiment proves, that the filaria does not pass from the living mosquito in the act of suction of water or sugar.

Whether the worm will pass into other kinds of food such as bananas, as has been suggested in connection with *Filaria immitis* by Grassi and Noé, is very doubtful. If the worm could pass into bananas and dates, it surely would do so in sugar.

*The daily examination of the water was made necessary in order to eliminate other organisms resembling the filariae that are likely to develop in water that has been standing some days.

This same series of experiments, and other observations, show that the filaria does not pass out at the time of laying the eggs.

There is one opportunity—the only one—for the filaria to find its way out of the mosquito into drinking water, and that is by accidental break in the integument; as may be shown experimentally in the act of dissection. But this must happen very rarely. Furthermore when the worms are set free in water they sink to the bottom and die by imbibition.

It appears then that only a series of accidents could bring the living filaria to the human stomach in the manner described.

The other hypothesis suggested to account for the transmission of the worm is that of a direct inoculation. There has been thus far no actual demonstration of the process.

Maitland argued that, "if it be true that the worm is introduced into the human body from the proboscis, we must suppose that the embryo must be always in position, ready to improve the opportunity given by the short time devoted to the act of suction." This is precisely what happens. The filaria is always ready.

The proof that the filaria remains day after day in the labium of the infected mosquito kept in captivity without a chance to repeat its feeding on blood, is found in the results obtained in sections by Low, and in the dissections made by James. These results suggested that the probable opportunity for escape is afforded in the act of drawing blood. The experiments here reported show that the migration does not occur during other acts of suction.

The experiments about to be described show how we may bring about at will the migration of the filaria, and permit the definite observation of the various steps in the process.

A mosquito is selected in which the filariae have reached their final stage of development, and are lodged in the labium. It is best to select an insect 28 to 30 days, or more, after infection. After removal of the wings and legs the insect is placed alive upon a slide and irrigated with a very weak salt solution. No cover-glass is employed. Under the microscope it is possible to see, through the transparent walls of the labium, the actively moving worms within that structure.

If no pressure* is used, these movements may be watched for hours without observing the escape of a single worm.

Usually all the setae are enclosed within the labium, thus preventing a clear view of the movements of the filaria. In order to obviate this difficulty I press very lightly with the side of a needle upon the base of the proboscis, and then with the point of a needle inserted between the setae and the labium, I pry them further apart. The filariae can now be distinctly seen actively moving and agitating the two tracheal tubes. The worms occupy usually the proximal third of the labium; sometimes they extend further out.

The slide is now placed near the flame of a Bunsen burner. This must be done very carefully in order to avoid fatal overheating to the insect and the parasite. Watching the preparation, an unusual activity in the movements of the filariae is now to be seen, and the cephalic end particularly becomes agitated as if seeking a point of exit. This cannot be found laterally because of the chitinous covering, and the filaria advances toward the anterior extremity of the labium. If the liquid is allowed to cool, the movements become slower and even cease altogether. Upon warming carefully again, and adding more tepid fluid, the movements are revived until the worms reach the point of the labium. If now the application of heat is stopped, we find that the worms appear to feel around with their cephalic extremity, but fail to break out.

I am sure that there is no natural orifice at the terminal end of the labium, because if there were, the filaria, having reached this point, would find no obstacle to its exit. And, furthermore, a careful study of the extremity fails to discover any orifice.

I cannot agree with Grassi and Noé, quoted by Dutton, who

*Bancroft, in experimenting with *Filaria immitis*; cut off and mounted on a slide under a cover-glass the proboscis of an infected mosquito. By making slight pressure upon the cover-glass he forced the setae out from the labium. He observed under the microscope that upon increasing the pressure the actively moving worm protruded from the extremity of the labium. "Whether there be at this point," says Bancroft, "a natural opening, appears to be doubtful; but every time that this experiment was tried the worm made its exit from the point and never elsewhere."

However, when we compress the labium, a rupture of its anterior extremity occurs, and the exit of a considerable amount of the fatty contents takes place. The break occurs at the point mentioned because of the thickness and unyielding character of the tissues at the end of the labellae. It is the break which offers a place of exit for the worms.

suggest that "when the mosquito, in biting, pierces the skin, there is a rupture at the point, through strain, and the filaria escapes. They imply that the crowding of the filariae causes the strain and distension of the tissues.

In my experiment there is no skin to pierce. Furthermore, the previous extrusion of the setae has really diminished the strain at this point. Nor can there be any tension when we have only one or two filariae with plenty of room. Besides, we do not have the bend or elbow formed by the labium in the act of biting, and which might contribute to the strain suggested by Grassi and Noé, for the labium lies perfectly straight. Even when the number of the filariae is great there is no irregular rupture anywhere in the labium, as would result from over tension.

If the heat is kept up carefully and steadily we can see the embryo seeking the point, making pressure there, and finally perforating the cuticle at a certain point.

This perforation is made quite suddenly, for we see the cephalic end jump out, as it were; the rest of the worm following slowly by serpentine movements. As soon as it reaches the fluid the serpentine movements continue, but the worm ceases to be able to advance. This remarkable difference in the results obtained by the movement indicates that the soft tissues at this stage are the natural element of the worm, and that it is prepared to move on into the human tissues directly from the mosquito. In water, on the other hand, the filaria not only cannot live, but cannot even move from place to place.

In their exit the worms follow the regular order in which they occur in the labium and head. We have seen two worms making their exit at the same time (Fig. 6).

Occasionally, after two embryos have been started out, it has been necessary to warm the liquid again in order to bring other worms down from the head to the labium. Of course, after the first embryo has broken out, the others find their way out with greater readiness.

What temperature is best adapted to stimulate the passing out of the filaria, I am as yet unable to say, but I am inclined to think that the temperature should be rather high. A slight vapor

should rise from the liquid, just enough to dim for a moment the lenses of the microscope.

Naturally enough the thought at once suggests itself of the importance of the heat in human blood as a factor in determining the exit of the filaria at the moment of biting.

POINT OF EXIT OF FILARIA.

Dutton describes a triangular space (seen in sections) near the point of the labium, and limited above by the chitinous band that forms the upper surface of the labium, and upon which the setae rest. He then adds: "This region appears to be the weak point in the chitinous exoskeleton of the labium; and it is probably at this point that the filaria escapes during the act of biting."

A careful study of the anatomy of the anterior extremity of the labium gives the following results:

The extremity of the labium presents an articulation indicated by a transverse groove (Fig. 5). The general appearance of this articulated end reminds us of the cloven hoof of certain animals. It is also divided symmetrically by a longitudinal groove, into two parts, called labellae. The separation, however, affects only the distal half of the end piece. In the proximal half the two sections are united by a membrane which, similar to an interdigital web, allows of free motion.

These two lateral bodies or labellae are cushioned with fatty tissue. The external (lateral) covering is chitinous and is rather thick. The internal (median) lining is a soft membrane covered with hairs.

I should mention that the whole inner lining of the labium is non-chitinous. It is a soft membrane made up of rounded cells. The filariae are lodged between this membrane and the outer cuticle.

When both labellae are united the longitudinal groove disappears and the whole structure appears as one piece.

Each labella has its own independent motion. When both segments are separated as far as the membrane (Fig. 5, B) will permit, we observe another complicated structure which protrudes pointlike between them, and forms with them a sort of tripod

(Fig. 5, a). This central portion appears to be a sort of buttress or rib in the interlabellar membrane. It consists of a strong chitinous groove (Fig. 5, b) along the median line, and two (c) lateral, hairy portions, of semicircular shape, partially covering the groove. Between the two lateral portions there is seen an obtuse point, which represents the protruding posterior wall. The whole structure, excepting the groove, is formed of a delicate hairy tissue.

It is difficult to understand the object of this median portion. The hairy covering suggests a tactile function; but it is also possible that the object of these structures may be to bring about a close approximation of the epi- and hypopharynx.

All these well cushioned structures of the labellae are evidently intended to hold together and control the other portions of the proboscis when they are being introduced into the skin, in the act of biting. The control thus exercised, permits a certain amount of sliding motion at the same time that the setae are held rigidly together. The terminal portion also covers up completely the spot where the lancets penetrate.

When we watch with a strong lens the act of suction, we observe at once the backward bend of the labium with the penetrating setae forming the chord of the arch. The bend or elbow formed by the labium becomes the more acute, the greater the depth to which the penetrating elements are introduced. If the depth be small, at least one-third (terminal) of the length of the labium remains closed, forming a cylinder; but if the insect is obliged to push deeply in search of blood then the whole labium as far as the transverse furrow enters into the formation of the arch, and the penetrating elements of the proboscis are held together solely by the labellae which bend downward and backward at the articulation, and are placed in a position parallel with the penetrating setae.

At all events the extreme end of the labium is applied in very close contact with the skin surface at the point of penetration of the setae.

The filaria makes its exit always from one of the lateral points, that is, from the tip of one or the other labella. At these points,

on the median aspect, the hairy integument is quite delicate, and readily perforated by the worm.

The place of perforation is, therefore, in close contact with the penetrating wound of the skin, and the setae. The latter are in constant motion (even movements of dilatation), and the best opportunity is offered for the successful inoculation of the parasite.

Having noted the extraordinary rapidity with which the filaria makes its exit, in one case five worms were seen to push their way out in less than one minute, it seemed important to determine the duration of the act of blood sucking. The minimum of time was found to be a minute and a half, and the maximum four minutes. The mean time was two minutes; or more than enough for the mosquito to discharge its whole load of filariae into the wound.

Fully understanding all these phenomena, we cannot help but be impressed with the facility with which filarial infection is brought about, when the conditions are favorable.*

Fortunately the non-experimental infections frequently miscarry through failure of perhaps the most insignificant detail in the evolution of the parasite.

EXPLANATION OF PLATES.

(Original drawings by the author.)

PLATE 1.

FIG. 1.—A. Embryo of *filaria sanguinis hominis nocturna* in human blood.

B. Sheath of the embryo in the same preparation.

FIG. 2.—The same embryo of Fig. 1 under the immersion lens. The transverse striation of the cuticle is distinctly seen.

PLATE 2.

FIG. 3.—1. Embryo in human blood—2 to 7. Stages of development with measurements of the embryo in the mosquito. The relative sizes represented in the figure are exact, and represent amplifications of 70 diameters. (a), (b), (c), Process of invagination in the tail end of the embryo, seen as the worm passes from the sharp (1st) to the appendicular (2^d) form.

FIG. 3'.—Anterior (a) and posterior (b) extremities of a fully developed embryo in the mosquito; (c) anus.

*I have gathered mosquitoes from the room occupied by the patient, the subject of my experiments, and I have found filariae in the process of evolution. It would be interesting to examine systematically the blood of those individuals who have lived in contact with the patient.

FIG. 4.—Peculiar formation seen in the caudal extremity of some embryos at the termination of the mosquito period of development; (a) anus; (b) posterior extremity where the three characteristic lobes present themselves; (c) projecting tip forming the end of the sleeve, (c); (d) portion of the sleeve that expands with the erectile movements of the same.

PLATE 3.

FIG. 5.—A and B. Extremity of the labium. Anatomic study to show the point where the filariae make their exit. In Fig. A the lateral portions (labellae) are in contact; but in Fig. B they are separated to show the special structure (a).

C. Special structure composed of (a), extreme blunt end; (b) chitinous groove; (c) lateral surfaces covered with fine hair.

FIG. 6.—Reproduction from nature of the exit of the filaria at the place of selection at the end of the labium.

PLATE 1.

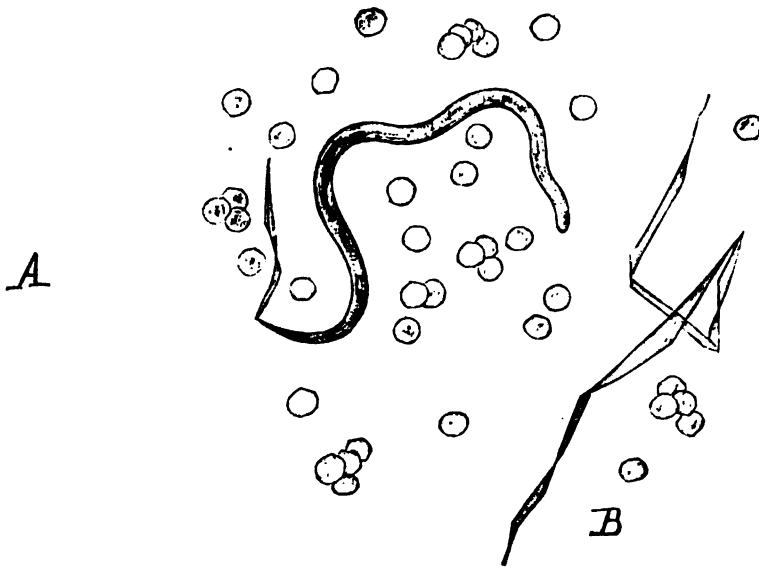


FIG. 1.

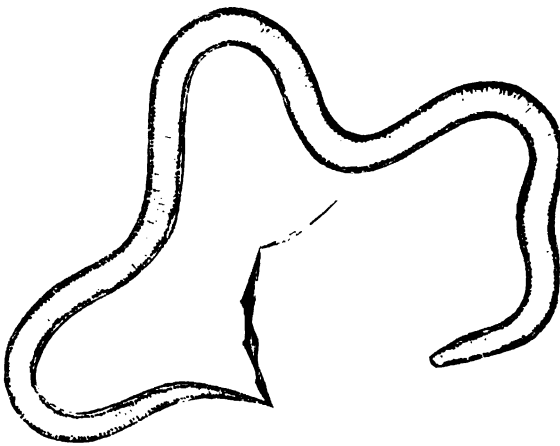


FIG. 2.

PLATE 2.

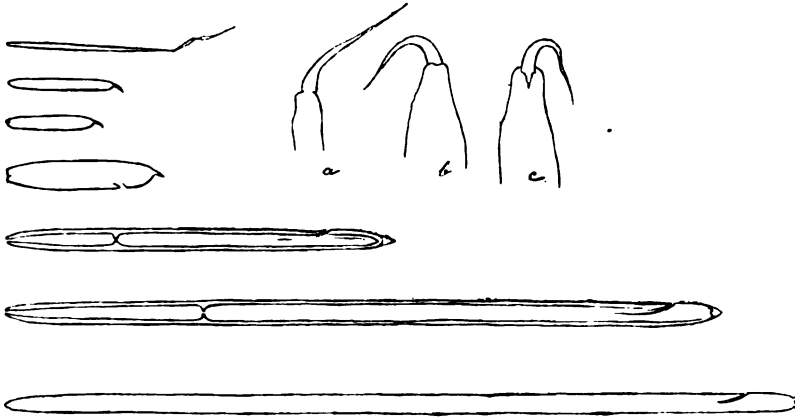


FIG. 3.

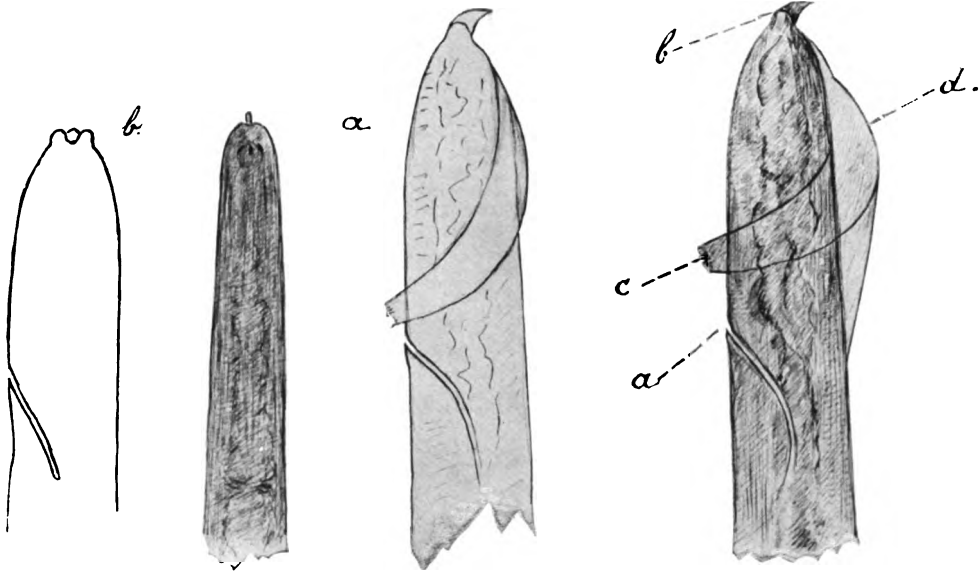


FIG. 3½.

FIG. 4.

PLATE 3.

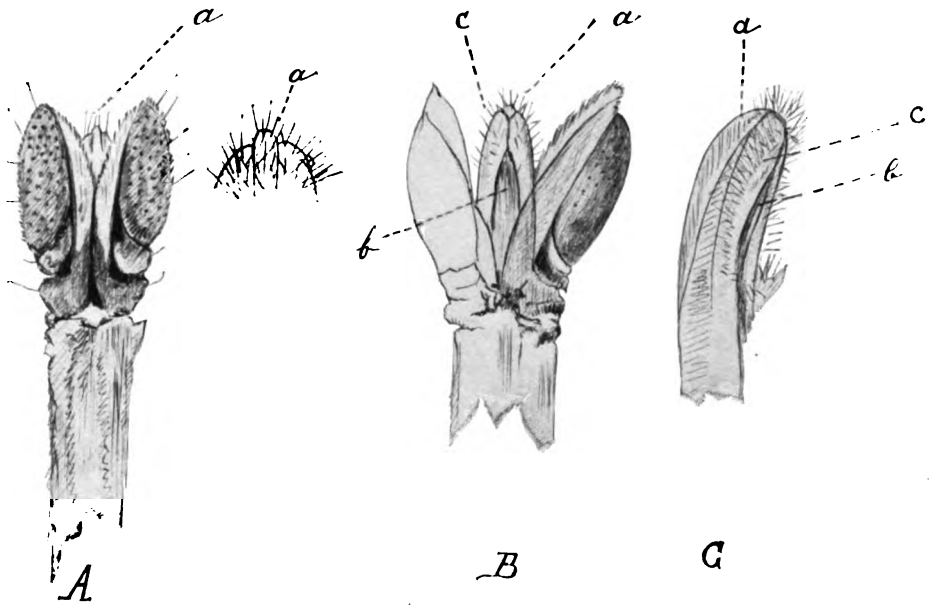


FIG. 5.

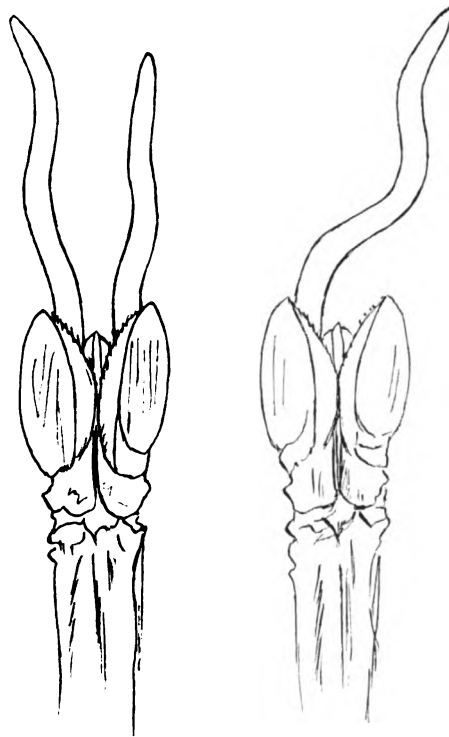


FIG. 6.

PLATE 3.

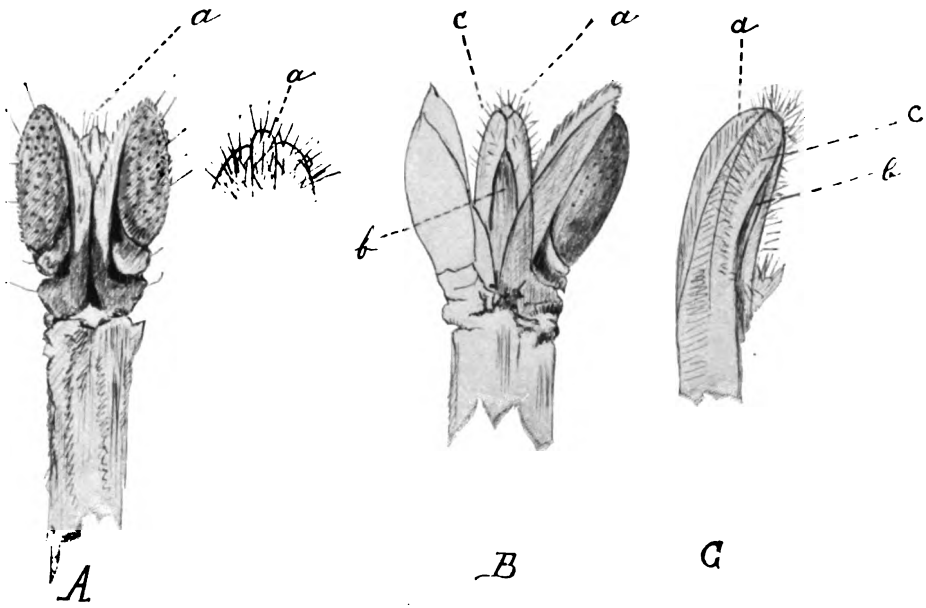


FIG. 5.

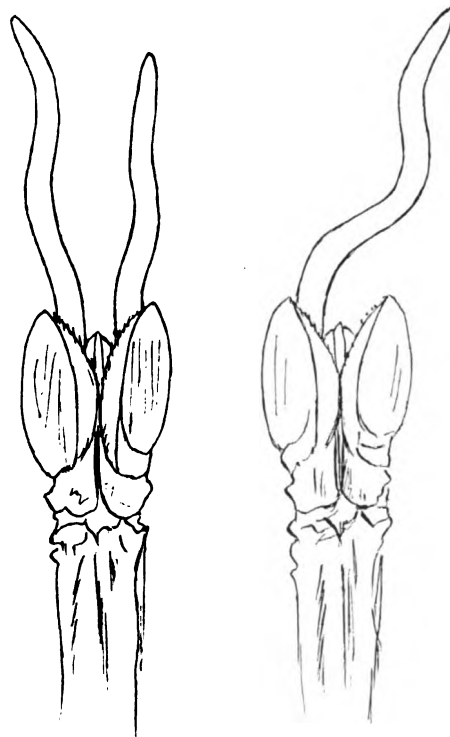


FIG. 6.

A POSSIBLE CAUSE OF THE FORMATION OF GAS IN CANS OF CONDENSED MILK.

CHARLES WRIGHT DODGE.

In the preparation of condensed milk it is sometimes found at the end of several weeks after the milk has been sealed up in the small tin cans that the ends of the latter bulge because of the formation of gas within the cans. Samples in this condition were received for examination with directions to pursue the investigation along bacteriological lines since experiments made by a competent person had shown the cause not to be chemical action.

The results which are summarized in this paper are derived from a study of the cause of this formation of gas.

When normal and bulged cans with their contents were compared it was found that while the interior of the former was bright and the milk had a normal taste and odor and gave only a faintly acid reaction with litmus, the inside of the latter was discolored and darkened, the contents having a cheese-like taste and odor and a strongly acid reaction. A preliminary test was made by inoculating fermentation tubes of neutral dextrose broth with milk taken from a badly bulged can, but no fermentation occurred. A test with milk from a sound can gave a similar result. On the supposition that the gas-forming organisms had perished in the former and were absent from the latter can, milk was used from one that showed only a slight amount of gas formation and from this four species of bacteria were isolated. These were tested singly and in combination with each other under a variety of circumstances and in numerous media, including diluted condensed milk, ordinary milk, lactose broth, dextrose broth, saccharose broth, and in combinations of these in different proportions with each other; in acid, neutral and alkaline media; at the room temperature and in the incubator; in the light and in darkness; in atmospheric air, in hydrogen and in carbon dioxide. Under none of these conditions did fermentation occur. Failing to find the offending organism among the four isolated, tests were

next made as mentioned above using the milk itself directly from a slightly bulged can, but no gas-formation took place. All tests were then repeated several times and were allowed to stand from two weeks to several months, but the results were always negative, and the conclusion was reached that bacteria are not the cause of the evolution of the gas.

By this time the supply of spoiled milk had become exhausted and recourse was had to butyric and lactic acids. It was learned that when dilute solutions of these in strengths varying from 1 in 200 to 1 in 500 in distilled water are allowed to act upon the metal of which the cans are made a slow evolution of gas takes place, its rapidity being inversely as the dilution of the acid. One-half c.c. of gas was formed at the end of two weeks in a tube of 15 c.c. capacity when a solution of 1 in 200 of lactic acid was allowed to work upon a piece of the tin having the diameter of a five-cent piece, the tin becoming discolored, of course, during the process. In greater dilutions the production of gas is, naturally, slower in rate and less in quantity. If the same action takes place in the cans of milk the bulging is easily accounted for, but the exhaustion of the supply of material renders conclusive proof impossible at this time. It is probable that in the instance cited the gas was formed not by the bacteria directly but by electrolytic action between the metals of which the cans were composed and the acids generated by the growth of bacteria in the milk before the latter was "condensed."

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